# POE AND POE-PEG-POE TRIBLOCK COPOLYMERIC MICROSPHERES FOR CONTROLLED PROTEIN DELIVERY

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# POE AND POE-PEG-POE TRIBLOCK COPOLYMERIC MICROSPHERES FOR CONTROLLED PROTEIN DELIVERY

BY

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# SUMMARY

Poly(ortho ester) (POE), poly(ortho ester) (POE)-poly (ethylene glycol) (PEG)-poly(ortho ester) (POE) triblock copolymers (POE-PEG-POE) and POE/PEG polymer blends with different PEG contents/molecular weights have been studied as the carriers for controlled protein delivery. Polymeric microspheres containing bovine serum albumin (BSA) were prepared using a double emulsion (water-in-oil-in-water) process.

Firstly, the fundamentals of the fabrication and characterization of POE-PEG-POE microspheres are reported. Since triblock copolymer is more hydrophilic than neat poly(ortho ester), it yields a more stable first emulsion (water-in-oil) and a greater BSA encapsulation efficiency. Uniform BSA distributions are observed within the microspheres by a confocal microscope. SEM pictures show that an increase in PEG content results in microspheres with a denser cross-section because of a more stable first emulsion and better affinity between the copolymer and water. POE-PEG(20%)-POE suffer significant swelling during the fabrication process and yields the biggest microspheres. Salt concentration in the external water phase significantly affects morphology of the resultant microspheres. Microspheres with a dense wall are produced when using pure water as the external water phase. Polymer concentration has less impact on BSA encapsulation efficiency but has a considerable effect on microsphere size and morphology. Increasing the concentration of the polyvinyl alcohol emulsifier does not cause an obvious decrease in microsphere size. However, an increased BSA loading results in bigger microspheres.

Following the previous research, the polymer erosion and the mechanism of protein release are studied. 14-week *in vitro* behaviours of POE-PEG-POE microspheres loaded with BSA have been monitored. SEM micrographs reveal that after 14-week incubation in PBS buffer, pH 7.4, 37°C, the polymeric particles remain spherical despite mass loss of almost 90%. On the other hand, molecular weight undergoes a high initial lose during the first two-week incubation for POE-PEG(5%)-POE and POE-PEG(10%)-POE. Then, it keeps relatively unchanged over 12 weeks. However, POE-PEG(20%)-POE copolymer provides a better compatibility between the POE and PEG blocks. Thus, its molecular weight remains relatively constant and mass loss shows quite sustained over the 14-week *in vitro* release. The similar phenomena are observed in the polydispersity index of the degrading copolymers. SDS-PAGE of the encapsulated BSA within the POE-PEG(5%)-POE microspheres displays that the structural integrity of BSA is intact for at least 8 weeks due to a mild environment provided by the copolymer. In addition, XPS and FTIR are utilised to investigate protein behaviours in the degrading microspheres. Protein release from the POE-PEG-POE microspheres shows a biphasic pattern, characterised by an initial stage followed by a non-detectable release. The non-release phase is dominated by either slow polymer degradation or dense microsphere matrix structures. The microsphere formulation is optimised and a sustained protein release over two weeks is achieved by using POE-PEG(20%)-POE at a high protein loading.

Modulation of BSA release from POE-PEG-POE microspheres has been investigated. Effect of PEG molecular weight and POE composition were studied. These results show that changing PEG molecular weight has little effect on the BSA release properties from POE-PEG-POE microspheres. Micropsheres made from POE-

PEG(Mn 1,000)-POE microspheres with more flexible POE block are investigated. More BSA can release from microspheres while higher initial burst and shorter release period (about 5 days) are introduced by this release system. Based on these studies, BSA release profiles could be optimised to a three-week nearly linear release.

On the other hand, POE/PEG blends of different PEG molecular weights and weight ratios have been utilized to alter the polymer degradation rate and therefore to modify the drug release kinetics from microspheres. BSA was encapsulated into POE/PEG blend microspheres using the W/O/W double emulsion technique. With the changing of PEG molecular weight and weight ratio, morphology, particle size, water uptake etc of the microspheres may be changed accordingly. Increasing the initial PEG content resulted in changing encapsulation efficiency, depending on the balance between polymer solution stability and water penetration. Changes of microspheres properties have greatly influenced the BSA release profiles from resultant microspheres.

# NOMENCLATURE

## Notation

| | |
|---|---|
| Mw | Molecular weight (weight average) |
| Mn | Molecular weight (number average) |

## Abbreviation

| | |
|---|---|
| ASF | Atomic sensitivity factors |
| BSA | Bovine serum albumin |
| CDM | trans-cyclohexanedimethanol |
| CLSM | Confocal laser scanning microscope |
| cP | Centipoise |
| DETUSO | 3,9-diethylidene-2,4,8,10-tetraoxaspiro{5,5} undecane |
| DSC | Differential scanning calorimetry |
| FTIR | Fourier transform infrared spectrophotometer |
| GPC | Gel permeation chromatography |
| HPSEC | High performance size exclusion chromatography |
| PBS | Phosphate buffer solution |
| PEG | Poly (ethylene glycol) |
| PLGA | Poly(lactic-co-glylic acid) |
| POE | Poly(ortho ester) |
| PrD | Propanediol |
| PTSA | p-toluenesulfonic acid |
| PVA | Poly (vinyl alcohol) |

| | |
|---|---|
| SDS-PAGE | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SEM | Scanning electron microscope |
| TEG | Tri(ethylene glycol) |
| TEG-diGL | Tri(ethylene glycol)-diglycolide |
| THF | Tetrahydrofuran |
| Tween 80 | Polyoxyethylene-sorbitan monooleate |
| UV | Ultraviolet |
| XPS | X-ray photoelectron spectroscope |

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# CHAPTER ONE

# INTRODUCTION

## 1.1 Scope of the Research

Successful drug therapy may be achieved in three ways: delivering the drug efficiently to the target; modifying the drug for increased efficiency; or finding a novel drug of inherently high efficacy. Of these three methods, to devise an efficient means of delivery is the most cost-effective one.

Conventional drug delivery systems have not incorporated the means of controlled release. The problem, however, is that after each dose, drug concentration immediately peaks and then declines rapidly. Taking four doses of a drug each day, for example, would yield a drug absorption pattern similar to Curve A of Figure 1.1. At times, the drug concentration is higher than the maximum level, leading to side effects. At other times, the concentration is too low to provide therapeutic benefits. It is desirable to release a drug at a constant rate, thereby maintaining drug level within the therapeutic window and reducing or eliminating side effects caused by over dosing.

Controlled drug delivery systems serve two functions. First, it involves targeted delivery of the drug to specific tissues or organs. The second function is to deliver the drug in a constant and therapeutic rate for a prolonged period of time. Thus, drug level in plasma could keep constant within the therapeutic window for a prolonged period of time (Curve B of Figure 1.1).

Since Yolls and his co-workers reported the use of the lactide-based copolymers for drug delivery in 1970, various polymeric controlled-release systems have been

developed. Such drug delivery systems have had an impact on nearly every branch of medicine including cardiology, endocrinology, oncology, ophthalmology and immunology. In the United States alone, annual sales of advanced drug delivery systems have exceeded $10 billion and are still rising rapidly. The world market of advanced drug delivery systems is currently valued at $50 billion a year, occupying 12.5% of world total pharmaceutical sales. By 2005, sales of delivery products are predicted to exceed $100 billion (*Informa Pharmaceuticals*). Investments in the "life sciences" industry in Singapore itself are expected to reach S$12 billion dollars by the year of 2005. This indicates an increasing support of "life sciences"-related research and development.

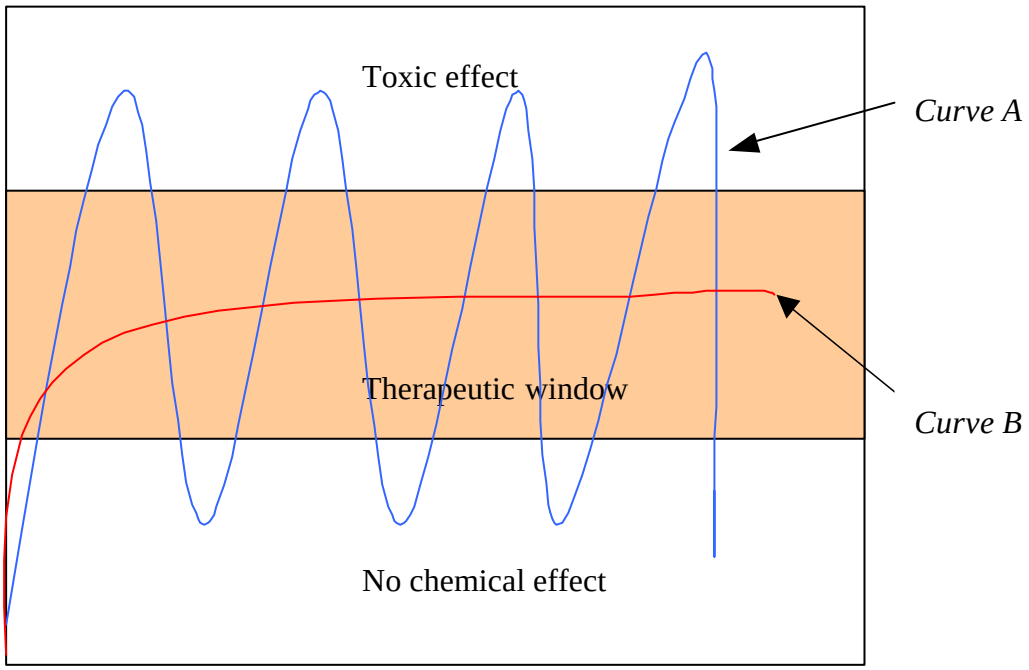



*Figure 1.1 Drug levels in plasma with traditional (Curve A) and advanced (Curve B) drug formulations.*

Currently, there are hundreds of protein drugs on the market or undergoing clinical trials, which include hormones, growth factors, cytokines, monoclonal antibodies, and

proteins for curing various diseases. All the protein drugs share three common problems: too large to be delivered through the skin; difficult to be administrated orally because of enzymatic degradation; unstable and short half-life *in vivo.* Therefore, protein drugs are required to be given either by constant infusion or frequent injections, which limits their acceptability by physicians and patients. Polymeric controlled release systems may provide a good approach to improving patients' compliance. Polymeric microspheres as a protein carrier have received increasing attention because they can be administrated by injection and protect proteins from degradation. My research is aimed at designing such a polymeric protein delivery system using a new generation of polymers.

## 1.2 Goals of the Present Work

In the next chapter, I present an overview of the fundamentals in controlled drug delivery including the different types of controlled release systems, drug release mechanisms, various polymers used, factors influencing their design and performance, the routes of administration, as well as likely future directions. This chapter also presents a review of some of the research work done in this area, in particular, the biomaterials being used and the problems associated with protein delivery. An understanding of this will therefore bring into focusing the objectives of my project. Chapter 3 details the methodology of this research project while the results obtained are presented and discussed in Chapters 4 and 5. A brief conclusion is presented in Chapter 6.

All the references cited and papers produced from this research work are listed at the end of the thesis as “REFERENCES” and “APPENDICE”, respectively.

# CHAPTER TWO

# THEORETICAL BACKGROUND AND RESEARCH REVIEW

In this chapter, I present an overview of the fundamentals in controlled release for drug delivery applications. Included here are some basic concepts in controlled release, different types of controlled release systems, various polymers used, factors influencing release system design and performance, the routes of administration, future directions, as well as my research objectives.

## 2.1 Drug Delivery

Oral administration and injection are conventional ways to administer a drug into human body. Compared to injection, oral administration is a more convenient route. However, the drug may suffer chemical and enzymatic degradation in the gastrointestinal tracts. In addition, the poor availability of drugs is another issue. For the injection route, although it is fast in action and has fewer absorption issues, patients usually suffer from pain caused by injection. Over the years, scientists have been striving to develop various delivery methods in order to increase efficacy of drug therapies and improve patient compliance. These methods include insulin pens [1], autoinjectors [2], mini-pumps, needleless injectors [3] and controlled drug delivery systems [4]. Of these methods, controlled drug delivery has attracted considerable attention. Various kinds of polymeric systems have been developed for controlled drug delivery.

## 2.2 Controlled Drug Delivery

In general, controlled drug delivery is classified into the following two categories.

- **Sustained release**: to sustain drug action at a therapeutic rate by maintaining a relatively constant, effective drug level in the body for a certain period of time.

- **Drug targeting**: to deliver drug to a specific tissue or organ using carriers or chemical conjugates.

In my report, if not mentioned, controlled release means sustained release. The aim of a controlled release system is to supply the optimal amount of a drug for a prolonged period of time than conventional dosage forms. For example, when the conventional tablet is taken repeatedly at fixed internals, its blood kinetics is characterized by the "Hill and Valley" phenomenon (Roseman, 1983) (Figure 2.1). The problem associated with this phenomenon is that the optimal concentration cannot be maintained and the peaks may occur above the toxic level or below the ineffective level. Furthermore, if a dosage is delayed or missed, additional difficulties may be incurred. Controlled release systems overcome these issues and provide many advantages over the conventional dosage forms.

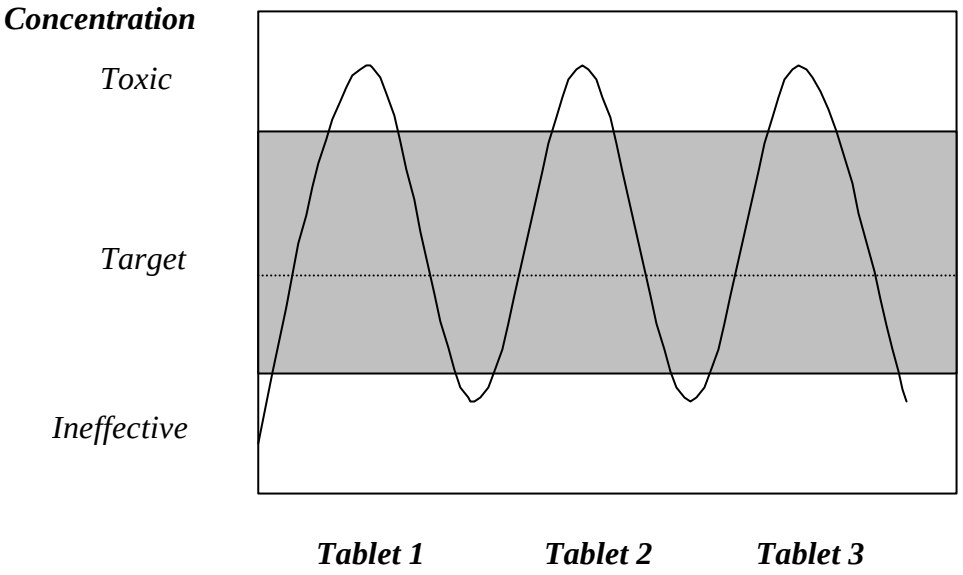



*Figure 2.1 "Hill and Valley" phenomenon of drug concentration when a drug is administered via conventional routes.*

Studies on controlled drug delivery systems were first focused on microencapsulation, which began in 1949 with the invention of Wurster process [5]. In 1953, the coacervation method was developed and led to the encapsulation of liquid substances. Between 1956 and 1966, over 50 patents were filed for microencapsulation techniques. Other important drug delivery systems such as implants and transdermal patches were reported between the mid-seventies and the early eighties. Since then, various advanced drug delivery systems have been developed for different applications.

## 2.3 Advantages of Controlled Drug Release

Controlled drug delivery systems could provide many advantages. A variety of controlled release systems such as Advil® tablets, sustained-release tablets containing

ibuprofen, and Nicoderm patches for nicotine addiction, have been commercialized. The main advantages that these systems provide include:

- **Increased effectiveness**

By sustaining the drug concentration in plasma for a prolonged period of time, increased effectiveness of the drug may be obtained. Because the drug is delivered more efficiently, smaller doses may be utilized. The side effects to the body could be minimized or eliminated.

- **Improved patient compliance**

Controlled release systems require fewer doses compared to conventional dosage forms. Therefore, they are more patient-friendly.

- **Lower cost**

Applying controlled release systems is more cost-effective because it needs less amount of the drug compared to conventional drug formulations.

Besides, to the commercial point of view, pharmaceutical companies are also increasingly looking into improving drug delivery as a way to gain a competitive advantage. Since developing a new drug is becoming more difficult and expensive, more and more pharmaceutical companies are now focusing on the improvement of drug delivery systems using the controlled release technology.

## 2.4 Drug Release Mechanisms Involved in Controlled Delivery Systems

An ideal controlled release mechanism for a drug delivery device is the one that exhibits zero-order release kinetics, that is, the release of drug is independent of time. However, with decreasing of drug level in the device, its release usually slows down. Thus, in most controlled release systems, drug release exhibits two phases: an initial phase and a second phase that relates to the rapid depletion of the drug from the device.

Basically, there are three primary mechanisms involved in the controlled release systems by which a drug can be released from a delivery system. They are diffusion, swelling followed by diffusion, and degradation.

Diffusion occurs when the drug travels through the polymer matrix into the external environment (Figure 2.2). The diffusion can happen on a macroscopic scale—as through pores in the polymer matrix, or on a molecular level, by passing between polymer chains.

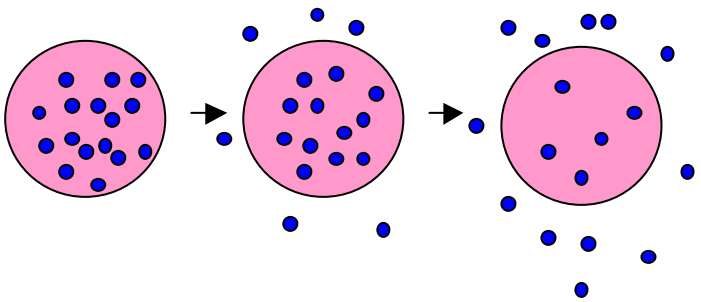


*Figure 2.2 Release mechanism 1: diffusion.*

In Figure 2.2, a polymer and a drug have been mixed to form a homogeneous system, also referred to as a matrix system. As the drug diffusion continues, its rate normally decreases, mainly because the drug has a longer distance to travel and thus requires a longer time to diffuse out of the polymer matrix.

For the diffusion-controlled systems, the drug delivery device is fundamentally stable in the biological environment and does not change its size either through swelling or degradation. In the diffusion-controlled systems, the polymer matrices must allow the drug to diffuse through the pores or macromolecular structure of the polymer into the biological environment without introducing any change in the polymer itself.

It is also possible for a drug delivery system to be designed so that it is incapable of releasing its agent until it is placed in an appropriate biological environment. Swelling-controlled systems are initially dry, after being placed in the body, they absorb water or other body fluids and then swell. Swelling increases the aqueous solvent content within the formulation and the polymer mesh size, enabling the drug to diffuse through the swollen network into the external biological environment. One example of these types of devices is shown in Figure 2.3. Most of the materials used in swelling-controlled systems are based on hydrogels. The hydrogels can absorb a great deal of fluid and, at equilibrium, typically comprise 60–90% fluid and only 10–40% polymer.

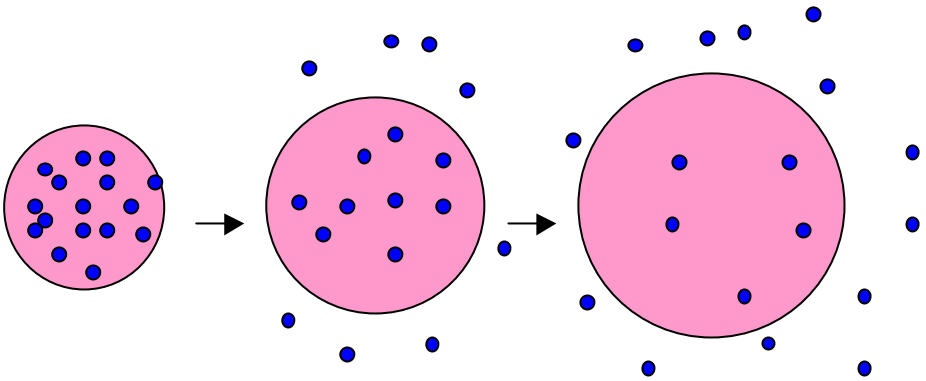


*Figure 2.3 Release mechanism 2: swelling.*

One of the most remarkable and useful features of polymer swelling is that swelling can be triggered by a change in the environment surrounding the delivery system. Dependent on the polymers used, the environmental change can involve pH, temperature, or ionic strength. The system can either shrink or swell upon a change in any of these environmental factors. For most cases, the structural changes of polymers are reversible and repeatable when additional changes in the external environment take place.

The controlled release systems described above are based on polymers, the chemical structures of which do not change during the course of drug release. However, a lot of attentions and research efforts are now concentrating on biodegradable polymers. These biodegradable materials can degrade within the body through natural biological processes, eliminating the need to be removed after the drug release has been completed. Figure 2.4 illustrates an example of degradation-controlled system.

Most biodegradable polymers are designed to degrade into smaller and biologically acceptable compounds as a result of hydrolysis of the polymer chains. For example, polylactides (PLA), polyglycolides (PGA) and their copolymers (PLGA) hydrolyze into lactic acid and glycolic acid, which further break down into carbon dioxide and water under physiological conditions. Degradation may take place through bulk hydrolysis, in which the polymer degrades in a uniform manner throughout the matrix. For some polymers, most remarkably, polyanhydrides and poly (ortho esters) (POEs), the degradation may occur only at the surface of the polymer matrices, resulting in a release rate that is proportional to the surface area of the drug delivery system [6].

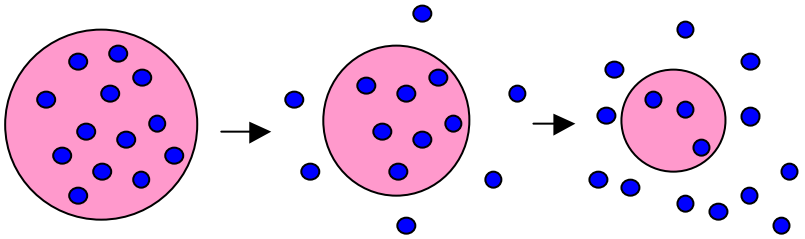


*Figure 2.4 Release mechanism 3: degradation.*

It was reported that the microspheres made from PLGA degraded through bulk hydrolysis in water or body fluids, yielding smaller and irregular forms over time [7]. In contrast, POE rods showed significant surface erosion after 9 and 16 weeks of implantation in rabbits, while the core of the matrices remained intact [8].

## 2.5 Factors Influencing the Design and Performance of Controlled Release Systems

To establish the criteria for design of controlled release products, a number of variables must be taken into considerations.

- **Drug properties:** the physicochemical properties of a drug, including stability, solubility, charge, and protein binding propensity etc., play an important role in the design and performance of controlled release systems.

- **Target sites:** in order to minimize side effects, it is desirable to maximize the fraction of applied dose reaching the target organ or tissue. This can be partially achieved by local administration or by the use of carriers. However, the absorptive surface of most routes is impermeable to macromolecules or other targeted delivery systems, thereby necessitating either intra-vascular or intra-arterial administration.

- **Route of drug delivery:** the area of the body in which drugs will be applied or administered can be restrictive on the basis of technological achievement of a suitable controlled release mechanism or device. At times, the drug delivery system, in certain routes of administration, can exert a negative influence on drug efficacy, particularly during chronic administration, and hence other routes of administration should be considered. Performance of the controlled release systems may also be influenced by physiological constraints imposed by the particular route, such as first-pass metabolism, GI motility, blood supply, and sequestration of small foreign particles by the liver and spleen.

- **Acute or chronic therapy:** consideration of whether one expects to achieve cure or control of a condition and the expected length of drug therapy are important factors in designing controlled release systems. Attempts to generate a one-year

contraceptive implant present significantly different problems in design from dosing an antibiotic for acute infection. Moreover, long-term toxicity of rate-controlled drug delivery systems is usually different from that of conventional dosage forms [9].

- **The disease:** pathological changes during the course of a disease can play a significant role in the design of a suitable drug delivery system. For example, in attempting to design an ocular controlled-release product for an external inflammation, the time course of changes in protein content in ocular fluids and in the integrity of the ocular barriers would have to be taken into consideration. Sometimes, one can take advantage of the unique manifestations of the disease state. For example, the higher plasminogen activator levels in some tumor cells can lead to preferential bioconversion of peptidyl prodrugs in these cells [10]. Similarly, the higher tyrosinase level in melanoma cells has been demonstrated to allow the preferential bioconversion of 2,4-dihydroxphenylalanine in them [11].

- **The patient:** whether the patient is ambulatory or bedridden, young or old, obese or gaunt, etc., can influence the design of a controlled release product. An implant or intra-muscular injection of a drug to a patient with little muscle movement may perform in a manner significantly different from that of an ambulatory patient. Some of these factors represent individual patient variation and cannot be controlled by the research scientists. For example, single unit controlled release products are particularly prone to intra- and inter- subject variation because of variability in individual GI motility [12].

From the previous discussion, it is clear that the formulation and performance of controlled release dosage forms depend on the physicochemical properties of the drug and its carrier. The pharmacokinetics and pharmacodynamics, to a large extent, are derived from the intrinsic properties of the drug. Thus, development and assessment of a controlled drug delivery system require a rather complete knowledge of the intrinsic properties of a drug and the ways in which it can influence the design of controlled release systems. In some cases, undesirable physicochemical and biological properties can be altered by suitable chemical modification, by use of a carrier, or perhaps by administration *via* another route.

## 2.6 Routes of Controlled Drug Delivery

Because of the significant advantages that controlled release systems provide, the list of controlled release products continues to grow, particularly in cost-neutral or cost-advantageous situations. There are some cases where controlled delivery is actually an enabling technology for a drug. For example, the drug is unstable or highly toxic without the controlled delivery devices. The most active areas for development, marketing, production, and sales of controlled delivery products are human pharmaceuticals, veterinary pharmaceuticals, cosmetics, and agricultural products.

Controlled release systems have already been found in a wide variety of applications in human medication, which have been delivered through various routes. Below is a brief description of some of these routes.

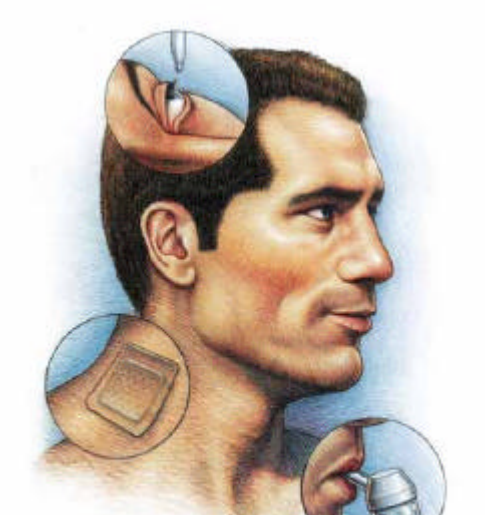


*Figure 2.5 Some routes of drug delivery.*

- **Oral delivery**

Oral administration is the most widely used route because of its simplicity and convenience. Problems, however, include the hostile environment of the gastrointestinal route as well as poor solubility of the polymeric materials in the various cellular barriers [13-14]. Adding to these constraints is the commonly substantial intra- and inter-subject variability associated with some of these factors. Generally, these factors cannot be controlled and hence severely limit the design of oral drug delivery systems.

Various approaches include using polymers that degrade preferentially in the colon, nanoparticles that can be taken up by intestinal Peyer's patches, and bioadhensives that interact strongly with intestinal mucosa. Hydrogels and osmotic pumps are two of the important classes of oral controlled-release dosage forms.

- **Transdermal delivery**

In the past, topically applied determatological drugs were used for localized treatment of skin diseases only. Recently, due to a better understanding of the anatomy and physiology of the skin as well as a more thorough understanding of percutaneous absorption, the limited permeability of human skin has also been utilized for systemic drug administration.

Transdermal patches, together with oral forms, are the most extensively developed because both transdermal and oral delivery are non-invasive. Almost all prescription medicines fall into these two categories. An advantage that transdermal delivery provides is that the drug enters the systemic circulation directly, avoiding liver metabolism and gastrointestinal incompatibility. Drugs such as scopolamine [15], nitroglycerin [16], and clonidine [17] have been administered *via* this route.

*Figure 2.6 A 3M â transdermal patch.*

- **Parenteral delivery**

Strictly speaking, parenteral products include all systems administered outside of the GI tract. However, parenteral routes are more commonly restricted to injectable administration such as subcutaneous, intramuscular, intraperitoneal, intrathecal, and

intraventricular injection. Generally, for life-threatening diseases or those in which the quality of life is drastically impaired, injectable controlled-release administration may be appropriate.

Currently, proteins are mostly administered *via* the parenteral route. Since proteins have short half-lives, efforts have been made to design controlled release parenteral delivery systems. Products based on PLGA and a small peptide, luteinising hormone and releasing hormone (LHRH) are on the market. More recently, microspheres containing recombinant human growth hormone have been developed and marketed successfully [18].

- **Pulmonary delivery**

Delivery of a drug to the respiratory tract for localized therapy of respiratory diseases is commonly accomplished *via* the airways because of their enormous surface area and accessibility [19]. Research has shown that many molecules are absorbed through the deep lung into the bloodstream without the need for enhancers used by other non-invasive routes. This high bioavailability makes the lung a natural target for peptides, proteins and small molecules. The structure of the lung is shown in Figure 2.7*.

Success of pulmonary delivery will depend on a number of factors. First, the drug used in aerosols must be quite potent but with negligible systemic side effects. Second, the drug must be able to gain access to its target site. Third, the drug must bind to tissue components thereby providing a high local concentration for prolonged

* From Chemtech 27 (2), pp34-38, 1997.

periods. Finally, good aerosol delivery from nebulizers is needed to enhance the amount of drug reaching the lung. Nevertheless, controlled delivery of drugs to the respiratory area is useful mainly for localized treatment of inflammation or cancer. It is unlikely that this route will supplant the oral or intravenous routes to achieve systemic effects.

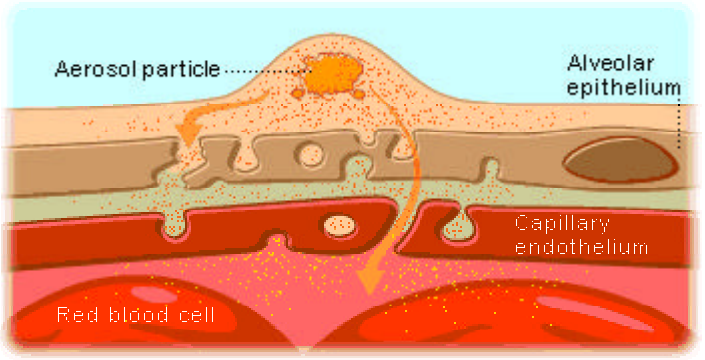



*Figure 2.7 Pulmonary delivery via alveolar site.*

- **Buccal/sublingual delivery**

Buccal drug delivery seems to combine the advantages of transdermal and oral delivery. Drug can be absorbed from the oral cavity through the oral mucosa either sublingually (under the tongue) or buccally (between the cheek and gingival). In general, rapid absorption from these routes is observed because of the thin mucous membrane and rich blood supply. For highly hydrophilic drugs, which also suffer from extensive pre-systemic elimination and require a rapid onset of action, sublingual or buccal administration may offer advantages over oral administration. After absorption, drug is transported through the deep lingual vein or facial vein. Thus, the buccal and sublingual routes can be used to bypass hepatic “first-pass” elimination.

Conventional buccal and sublingual dosage forms are typically short acting because of limited contact time between the dosage form and the oral mucosa. Since sublingual administration of drugs interferes with eating, drinking, and talking, this route is generally considered unsuitable for prolonged administration. Compounds administered by either the buccal or sublingual route include steroids, barbiturates, papain, trypsin, and streptokinase-streptodornase [20].

## 2.7 Controlled Delivery Devices

In recent years, there have been numerous developments in polymeric carriers for controlled drug delivery. A few examples reported in the literature include:

- Films with a drug *in* a polymer matrix (monolithic devices) [21-23];
- The drug contained *by* the polymer (reservoir devices) [24];
- Polymeric colloidal particles or microencapsulates (microparticles, microspheres or nanoparticles) in the form of reservoir and matrix devices [22, 23];
- The drug contained by a polymer containing a hydrophilic and/or leachable additive such as a second polymer, surfactant or plasticizer to give a porous device, or a device in which the drug release may be osmotically controlled (both reservoir and matrix devices) [25-28];
- Enteric coatings that are ionized and dissolved at a suitable pH [26];
- Soluble polymers with covalently attached pendant drug molecules [29-33];

- Devices such as osmotic pumps where drug release rate is controlled dynamically [34].

### 2.7.1 Reservoir devices

A typical approach to controlled release is to encapsulate or contain a drug entirely within a polymer film or coat (e.g. microcapsules or spray/pan coated cores). Film coatings have been reviewed by Kala *et al.* [35], and microencapsulation has been summarized by Arshady *et al* [36, 37].

Various factors such as additives, polymer functionality, porosity and film casting conditions that can affect the diffusion process should be taken into considerations for the design of desired reservoir devices.

When the device contains a water-soluble drug, the release rate of the drug decreases with time as the drug concentration, the driving force for the drug release, within the device decreases. However, if the drug is in a saturated suspension, the driving force is kept constant until the device is no longer saturated. Thus, zero order release is achieved [38, 39]. Alternatively, the drug release may undergo a desorption-controlled mechanism and it is then a function of the square root of time.

Transport properties of coated tablets can be enhanced compared to tablets without a polymer film since the coating layer may help build up an osmotic pressure that may accelerate drug release [28].

### 2.7.2 Monolithic devices

Monolithic devices are possibly the most common drug delivery systems. This is because they are easy to fabricate compared to reservoir devices. In addition, there is no danger of an accidentally high dosage that could result from the crack of the membrane of a reservoir device. In such a device, a drug is dispersed within a polymer matrix. Monolithic devices are normally formed by the compression of a polymer/drug blend or by dissolution or melting.

### 2.7.3 Other Types of Controlled Release Devices

#### 2.7.3.1 Pendent devices

Scholsky and Fitch (1986) [40] developed a means of attaching a variety of drugs such as analgesics and antidepressants through an ester linkage to poly (acrylate) ester latex particles prepared by aqueous emulsion polymerization. These latices could self-catalyze the drug release by hydrolysis of the ester linkage when they passed through an ion exchange resin so that the polymer end groups were converted to their strong acid form. [29, 31]

#### 2.7.3.2 Enteric films

Enteric coatings consist of pH-sensitive polymers. Typically the polymers are carboxylated and can hardly dissolve in water at low pH, while at high pH the polymers are ionized, resulting in swelling, or dissolving of the coatings. Coatings can therefore be designed to remain intact in the acidic environment of the stomach but to

dissolve and release their contents rapidly when they reach the more alkaline conditions of the upper intestines. For example, EUDRAGIT®L 100, a product of Röhm Pharma Polymers, is a pH dependent anionic polymer powder solubilizing at above pH 6.0 for targeted drug delivery in the ileum.

#### 2.7.3.3 Osmotic pumps

Osmotic pumps contain an osmotic agent (*e.g.*, a drug in a salt form), which absorbs water from the surrounding medium *via* a semi-permeable membrane. Such a device called the elementary osmotic pump, was described by Theeuwes *et al* (1975) [34]. Figure 2.5 illuminates an example of osmotic pumps. The active agent serves as the osmotic agent, which is surrounded by the rigid semi-permeable membrane containing a delivery orifice. When the pump is immersed into an aqueous medium, pressure is generated within the device and drives the drug out of the device through an orifice. Since the internal volume of the device remains constant and there is an excess of solid drug in the device, the drug release rate remains constant.

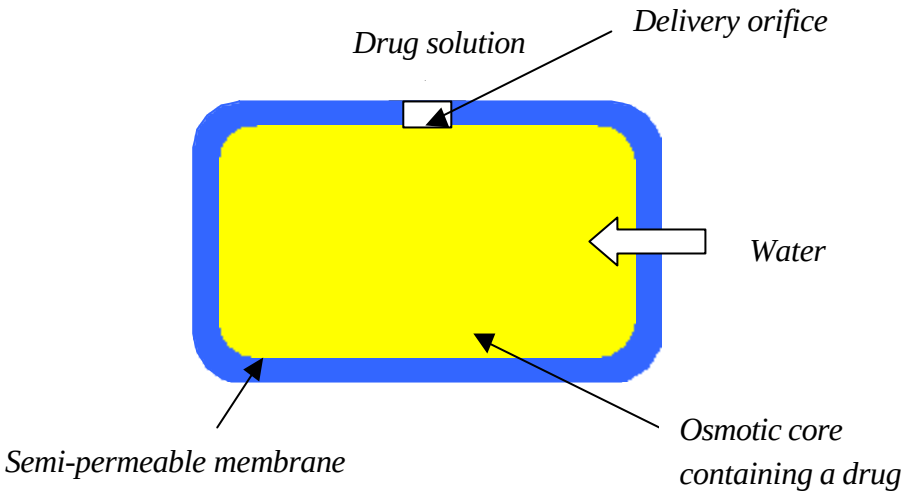



*Figure 2.8 Cross-section of an elementary osmotic pump.*

#### 2.7.3.4 Electrically stimulated release devices

Yuk *et al.* [41] prepared monolithic devices using polyelectrolyte gels that swelled when an external electrical stimulus was applied. The drug release could be modulated by changing the current, giving a pulsatile release profile.

#### 2.7.3.5 Hydrogels

Hydrogels are not only used as a drug carrier but also find a number of applications in bioengineering (e.g. soft contact lenses and various soft implants) [42, 43]. Recently, stimuli-sensitive hydrogels have attracted considerable attention, which could respond to small external stimulus changes, such as temperature [44] (Figure 2.9), pH [45], photo field [46] and antigen [47]. This unique responsive property makes this hydrogel especially useful for biomedical and bioengineering applications such as

protein-ligand recognition [48], on-off switches for modulated drug delivery [49-51] or artificial organs [52] and immobilization of enzyme [53].

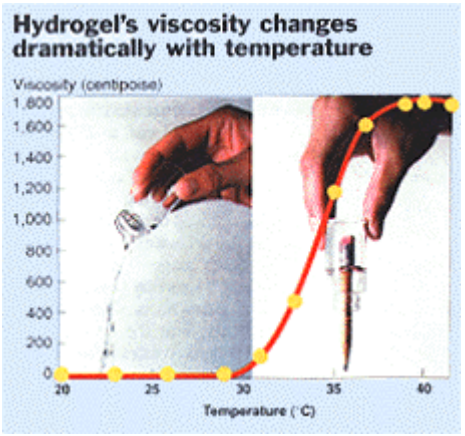



*Figure 2.9 Changing of viscosity of hydrogel with increased temperature.*

In the following paragraphs, polymeric microspheres systems for controlled drug delivery will be introduced. Section 2.9 will provide an overall view of the polymers used in controlled drug delivery systems.

## 2.8 Drug Delivery Based on Polymeric Microspheres

### 2.8.1 Background - Microencapsulation

Microencapsulation is one of the most intriguing fields in the area of drug delivery. It requires knowledge of polymer science, familiarity with emulsion technology and an in-depth understanding of drug and protein stabilization. Some journals are now solely dedicated to the area of microencapsulation (e.g. *Journal of Microencapsulation*). Although scientists at the beginning of the 1970s were primarily concerned with the encapsulation of dyes to produce carbonless paper,

today the technology has been mastered to such a level that cells as well as delicate proteins and genes can be encapsulated.

### 2.8.2 Microspheres

Microspheres are produced by a kind of microencapsulation technique. Theoretically, microspheres are spherical particles with a size range from 1 to 1000 μm. An active ingredient may be dispersed in a hydrophilic or non-biodegradable matrix, or may be enveloped by a release-modulating polymer layer. Figure 2.10 shows a cross-section image of a single microsphere.

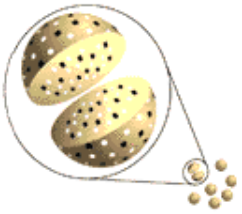


*Figure 2.10 Cross-section of a microsphere.*

The microsphere system is suitable for oral, pulmonary and injection administration. It is a good approach to delivering proteins through parenteral routes [54, 55]. Among the various biodegradable polymers such as PLA, PGA, PLGA, polyanhydrides, and POEs, PLGA has been widely utilized for microspheres fabrication for controlled delivery of peptides, native and synthetic proteins and lately, nucleic acids. The PLGA microspheres have a size ranging from 1 to 250 μm. Many factors are important to formulate this system: 1) polymer molecular weight, monomer ratio and morphology, which determine drug release rate; 2) particle size, which is important in terms of interaction with phagocytic cells; 3) safety, which is related to the *in vivo* polymer degradation, and 4) stability, both in storage and in the biological fluids.

### 2.8.3 Microspheres preparation --- a double emulsion process

There are several ways to prepare microspheres, including single/double emulsion, spray drying and other processes. Among these methods, the water-in-oil-in-water (W/O/W) double emulsion technique is the most commonly used one. The choice of a method depends on the physical and chemical properties of the drug as well as the polymers.

A typical W/O/W double emulsion process for microspheres fabrication is shown in Figure 2.11 [84]. The inner aqueous phase containing a hydrophilic drug is emulsified with an immiscible polymer solution by sonication to produce a primary emulsion. This first emulsion is homogenized with an aqueous solution containing a surfactant to yield a W/O/W double emulsion. Water is added to the double emulsion with stirring to extract the solvent from the oil droplets to the external water phase. After that, the microspheres are filtered, washed with water and then dried. The composition of the aqueous phase, polymer concentration as well as the surfactant applied play an important role in the pattern of drug release since porosity and water permeability of the resultant microspheres depend on these factors. The W/O/W double emulsion method is suitable for the encapsulation of hydrophilic drugs. However, it has also been employed to produce lipophilic molecules-containing polymeric microspheres [56].

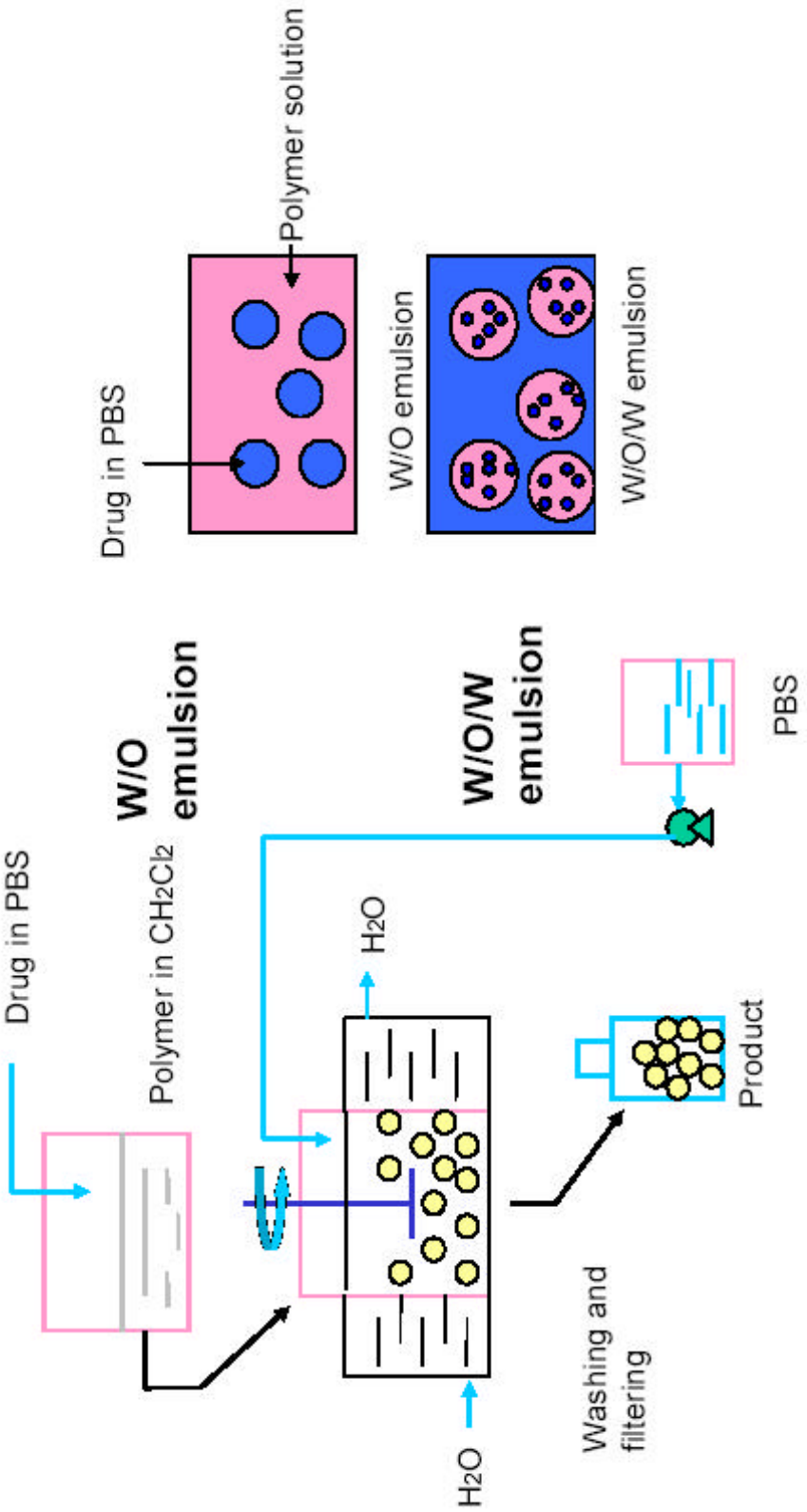



*Figure 2.11 Microsphere fabrication using W/O/W double emulsion technique.*

## 2.9 Polymers Used in Controlled Drug Delivery Systems

In a polymeric drug delivery system, the polymer is used as a protectant of a drug during the drug's transfer through the body until it is released, or used to control the release rate of a drug. Selecting a suitable polymer is critical to the design of a desired controlled release system.

### 2.9.1 Polymer characteristics

There are three characteristics that describe an ideal polymer used for drug delivery. First, the polymer must be biocompatible and degradable. This means that the polymer should degrade *in vivo* to smaller fragments that can then be excreted from the body. If a polymer matrix fails to degrade inside the body, then it must be surgically removed after the drug is released. Second, the degradation products of the polymer must be nontoxic and not triggering any inflammatory response. Third, the degradation of the polymer should occur within a reasonable period of time.

### 2.9.2 Biologically degradable polymers

Biologically degradable polymers are defined as a class of polymers that can degrade into smaller segments due to the presence of chemicals inside the body. Biodegradable polymers and bioabsorbable polymers are the two kinds of degradable polymers that have the ability to break down in this manner. Biodegradation can be described as degradation that is promoted by enzymes. In other words, enzymes that

are present within the body break down polymers. Bioabsorbable polymers are those that are degraded by other chemicals in the body. Both biodegradable and bioabsorbable polymers have been used for controlled drug delivery.

Biologically degradable polymers include natural, modified natural and synthetic polymers. Collagen, cellulose, and chitosan are examples of natural polymers. Natural polymers have been tested as drug delivery matrices for the delivery of protein based drugs. Modified natural polymers are natural polymers that are altered in order to suit a particular application. The reason for modification is that these polymers often take longer time to degrade within the body. By adding polar functionalities to the polymers, the problem could be overcome since the polar groups are more flexible and can therefore promote the degradation of the polymers.

The addition of functional groups may change the physical and chemical properties of the polymers. In the process of modifications of natural polymers, the nature and extent of modification should be considered. If a polymer is modified in excess, the natural polymer may not degrade easily. In addition, the added functional groups may be converted to toxic degradation products.

Over the past few decades, synthetic polymers have been actively studied for use in drug delivery systems. Polyesters, polyurethanes, polyanhydrides and poly (ortho esters) are the most popular examples.

### 2.9.3 Classification of synthetic biodegradable polymers

In broad terms, synthetic polymers may be classified as either biodegradable or non-biodegradable. Biodegradable polymers have recently captured much attention mainly because non-biodegradable systems need retrieval or further manipulation after introduction into the body.

In the realm of biodegradable polymers, there exists another level of classification based upon the mechanism of polymer erosion. Two erosion mechanisms can be identified, including surface and bulk erosion. In practical terms, these two mechanisms represent extremes. For most biodegradable polymers, both mechanisms will occur. However, the relative extent of surface or bulk erosion varies depending on the chemical structure of the polymer backbone.

Surface erosion occurs when the rate of polymer erosion is equal to or exceeds the rate of water permeation into the bulk of the polymer. This is often considered to be a desirable mechanism in drug delivery because the kinetics of erosion, and hence the rate of drug release, is highly reproducible. In an ideal surface erosion process, the erosion rate is directly proportional to external surface area. Surface erosion can lead to zero-order drug release as long as diffusional release is limited and the overall shape remains constant.

Bulk erosion occurs when water molecules are able to permeate into the bulk of the polymer matrix at a faster rate than erosion. As a consequence, polymer molecules in the bulk may be hydrolyzed and the kinetics of polymer degradation/erosion is more

complex than surface eroding polymers. The majority of biodegradable polymers used in controlled drug delivery undergo bulk erosion, including the very important materials, poly (esters).

Hopfenberg [57] derived expressions for drug release from erodible slabs, cylinders and spheres. If the erosion rate is proportional to the continuously changing surface area of the device, the equation (2.1) for drug release could be

$$\frac{M_t}{M_\infty} = 1 - \left[1 - k_0 t / C_0 a\right]^n \qquad (2.1)$$

*where* $Mt$ = *mass released at a given time t*

$M¥$ = *initial incorporated mass*

$k_0$ = *erosion constant*

$t$ = *time*

$C_0$ = *initial uniform concentration of drug in matrix*

$a$ = *radius of sphere/cylinder or half-thickness of a slab*

$n$ = *3 (sphere), 2 (cylinder), 1 (slab)*

Figure 2.12 illustrates the classification of polymers used in controlled drug delivery systems.

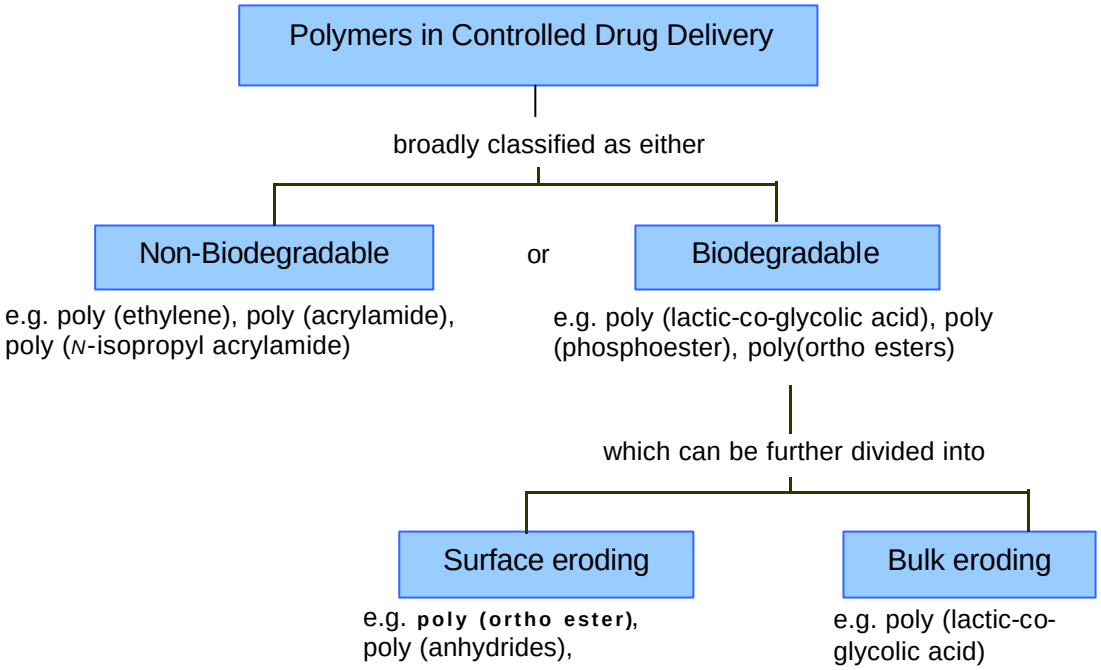



*Figure 2.12 Classification of polymers used in controlled drug delivery.*

### 2.9.3.1 Poly (esters)

Poly (esters) are the most widely studied biodegradable materials, including PLA, PGA, and PLGA (Figure 2.13).

The degradation mechanism of poly (esters) is classified as bulk degradation with random hydrolytic scission of the polymer backbone.

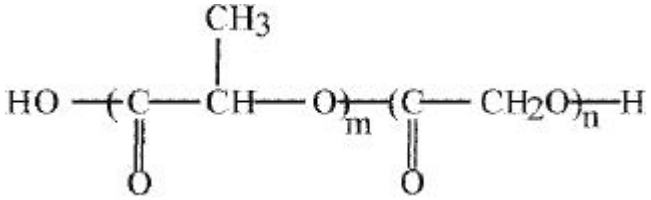


*Figure 2.13 Chemical structure of PLGA.*

#### 2.9.3.2 Poly (ethylene glycol) block copolymers

Poly (ethylene glycol) (PEG) is an outstanding biocompatible material. One of the emerging uses for inclusion of PEG in a controlled release system arises from its interaction with protein. Conjugation of proteins with PEG has proved to be able to provide prolonged protein circulation life, reduced immunogenicity and antigenicity [58]. PEG chains at the surface provide the incorporated substance longer circulation time in the body by prolonging biological events such as endocytosis, phagocytosis, liver uptake and clearance, and other adsorptive processes. [59-63]

PEG can be made with a range of terminal functionalities, which lead to its easy incorporation into copolymer systems. PEG is commonly terminated with chain-end hydroxyl groups that provide a ready handle for synthetic modification. Diblock PLA-PEG and triblock PLA-PEG-PLA systems have been synthesized and characterized with various PLA contents [64-68]. Jeong et al. prepared thermo-sensitive PLA-PEO hydrogels that exhibit temperature-dependent gel-sol transition for use as injectable drug delivery systems. [69]

#### 2.9.3.3 Poly (ortho esters)

The motivation for designing poly (ortho esters) for drug delivery was the need to develop biodegradable polymers in which drug release exhibits surface erosion mechanism. [70]

Most studies on poly (ortho esters) have focused on the synthesis of polymers by the addition of polyols to diketene acetals. For example, Heller et al. [71] have described the synthesis and application of the 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5,5] undecane (DETOSU)-based poly (ortho esters). The basic structure is formed by the addition of the DETOSU monomer to a diol to form the chemical structure shown in Figure 2.14.

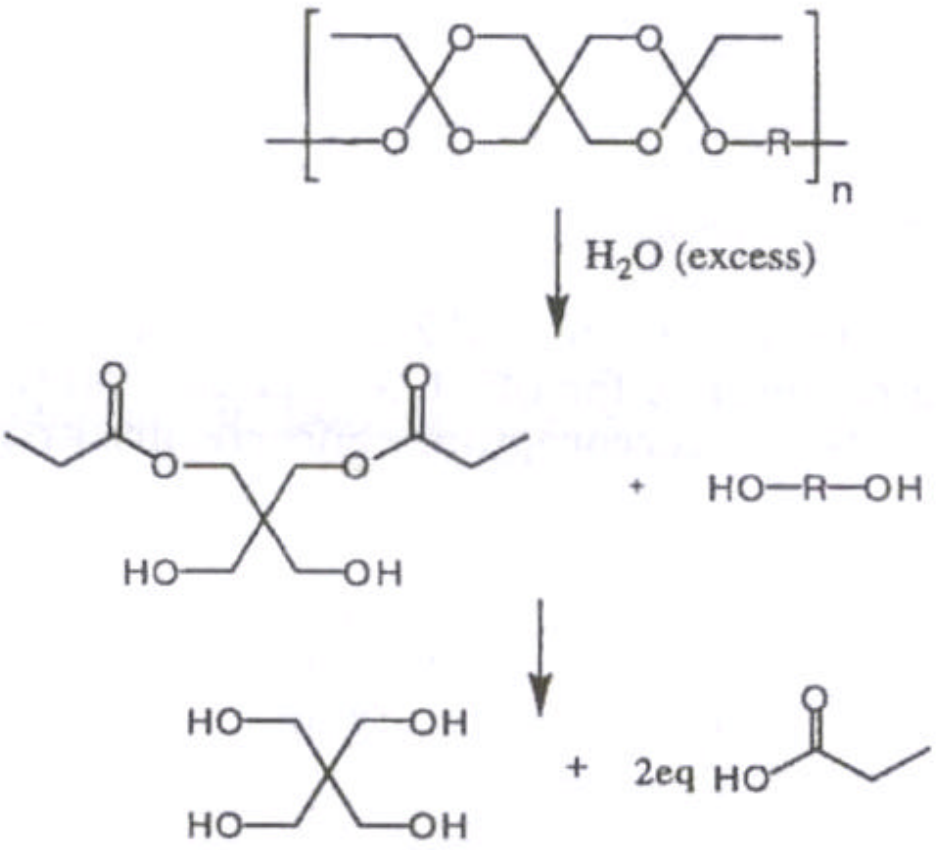



*Figure 2.14 Degradation of the 3,9-bis (ethylidene-2,4,8,10-tetraoxaspiro[5,5]undecane) (DETOSU)-based poly(ortho esters).*

The DETOSU-based poly (ortho esters) contain acid labile ortho ester linkages in their backbone. In aqueous environments, the ortho ester groups are hydrolysed to form pentaerythritol dipropionate and diol monomers (Figure 2.14). The pentaerythritol diopropionate is further hydrolyzed to pentaerythritol and acetic acid.

The acid-catalyzed hydrolysis of POE can be modulated by introducing either an acidic or a basic excipient into the matrix system. The rate of hydrolysis is increased by the addition of acidic excipients [72]. Alternatively, basic excipients stabilize the bulk of the matrix but diffuse out of the surface region, thereby facilitating surface erosion [73]. This approach has been employed in the temporal controlled release of tetracycline over a period of weeks in the treatment of periodontal disease [74].

Recently, a number of changes in diol structure have been attempted to avoid the need for acidic excipients. These new poly (ortho ester) structures address the problem of acidic excipient diffusion from matrices, which leads to unpredictable degradation kinetics. Ng et al. described the synthesis of self-catalyzed poly (ortho esters) that contain glycolide segments [75]. Once glycolide segments degrade, its degradation products catalyze ortho ester bond breakage, hence forming a self-catalyzing system. The synthesis of these polymers is shown in Figure 2.15.

A useful feature of the DETOSU systems is the ability to control the mechanical properties by changing the diol monomer ratios within the final polymeric structure. For example, Heller et al. have shown that the glass transition temperature of polymers containing a rigid diol monomer (trans-cyclohexanedimethanol) (CDM) and a flexible monomer (1,6-hexanediol) could be varied between 20 and 105°C by increasing the proportion of the rigid diol [70]. This control can also be achieved with the glycolide-containing polymers [75].

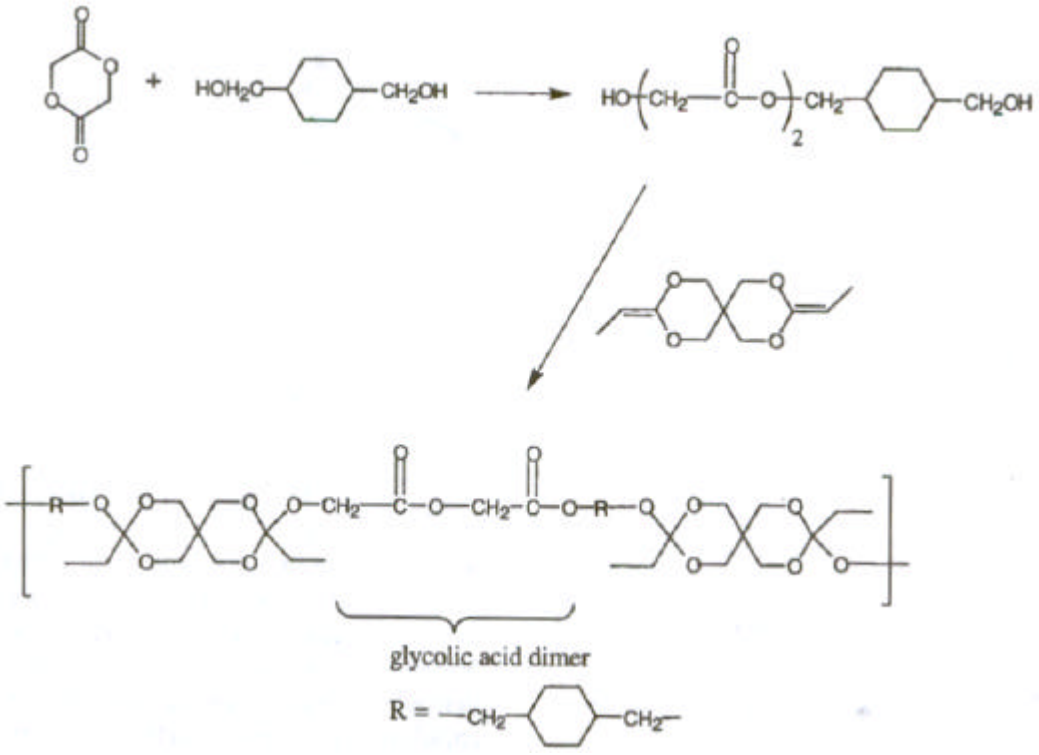



*Figure 2.15 Synthesis of a self-catalyzed poly (ortho ester) containing glycolic acid dimer.*

#### 2.9.3.4 Polymer Properties Influencing Drug Release

Drug release from a polymeric matrix is affected by various factors including drug nature, polymer properties and morphology of the matrix. Two important polymer properties that influence drug release are described below.

a) Molecular weight and molecular weight distribution. Molecular weight reflects the size and size distribution of a polymer. While we may expect qualitatively that drug diffusion rate decreases with increasing molecular weight, the critical factor that governs drug diffusion is determined by the

overall microstructure of the polymer in the presence of diffusing and other foreign species.

b) Transition temperature. The glass transition temperature can be modified by changing the strength of the secondary forces within the polymer. This can be accomplished by introducing an additive, such as a plastizer, to the polymer. Another approach to modifying the glass transition temperature is to form a copolymer. Drug transportation is modeled by a migration of the penetrant through holes or free volume within the polymer. Therefore, the rate of transport is based on the probability of creating a hole of sufficient size to accommodate the penetrant, and the probability for this penetrant to have sufficient energy to enter this hole. At temperatures significantly higher than the Tg, both probabilities must be taken into account to estimate diffusivity. However, near the Tg, the amount of free volume is small, so the probability of encountering holes of sufficient size dominates mass transfer. In most cases, when the temperature is lower than the glass transition temperature, the amount of free volume is small, and the redistribution of holes within the polymer is negligible because segmental motion is virtually nonexistent.

#### 2.9.3.5 Factors to Be Considered in Selecting A Polymer for Controlled Drug Delivery

There are several important factors to be considered in selecting or developing a polymer for controlled drug delivery:

- Biocompatibility and toxicity
- Regulatory acceptance or concerns
- Degradation rate, degradation products, and biocompatibility as well as toxicity of the degradation products
- Cost
- Chemical, physical, and mechanical properties
- Processing requirements
- Compatibility of the drug with the polymer
- Required sterilization methods
- Glass transition temperatures

The relative importance of these factors varies depending on the particular application. For example, if the product contains a thermally sensitive active agent, a polymer requiring thermal processing might not be acceptable.

## 2.10 Protein Delivery and a Model Protein - Bovine Serum Albumin (BSA)

Efficacious delivery of proteins and peptides is taking on increasing significance as biotechnology companies mature and begin commercializing their products. As a result, there has been increasing interest in the development of protein and peptide delivery systems. The high level of interest is attributed to several factors. As the pharmaceutical and biotechnology industries consolidate and mature, there is an interest in value-added technologies, which drug delivery systems can provide. There

is also an impending stream of patent expirations, which is fueling the demand for the rapid development of new products that may be realized by drug delivery technologies.

Proteins are polymers made up mainly by amino acids, which polymerize to give long chains that then fold into a functional 3-dimensional structure. Proteins must maintain this structural and chemical integrity to function properly. This 3-dimensional structure, however, is held in place by weak forces that can be easily broken. Once this configuration is destroyed, the proteins lose their activity, and are essentially denatured. Proteins can lose their activity easily when subjected to adverse conditions such as high temperature, mechanical agitation and extreme pH etc.

For a long period of time, controlled release devices were only capable of slowly releasing drugs of only low molecular weight (<600). Large molecules such as proteins were considered too large to slowly diffuse through most of the polymeric materials. It could diffuse through highly porous membranes, e.g. Millipore filters or certain gels such as polyacrylamide. However, the diffusion was too rapid and could result in tissue damage. The discovery that matrices of solid hydrophobic polymers enable molecules of any size to be released for over 100 days allows the controlled delivery of a variety of proteins. Non-degradable ethylene-vinyl acetate and degradable lactic-glycolic acid copolymers are materials that are capable of performing the task. Certain hydrogels such as poly (hydroxyethylmethacrylate) or poly (vinyl alcohol) are also effective but they release proteins in a shorter period of time.

There are still problems facing protein delivery. The most significant issue is the denaturation, degradation and aggregation of the encapsulated proteins when they remain in the body for long time. It can cause a loss of biological activity as well as changes to its immunogenicity. To circumvent these problems, several approaches have been explored in order to protect proteins from aggregation and degradation using stabilizing excipients [76-78]. Protein stabilization is one of the most important issues, which the researchers must take into considerations during the design of controlled release systems.

BSA is a commonly used model protein during the development of controlled release systems [79]. It is known for its surface-active property, which is responsible for its deposition at the oil-water interface [80]. This has a strong stabilizing effect on the first emulsion [81] in the double emulsion (W/O/W) process and leads to the formation of a matrix-like structure.

BSA in a solid form is stable for about three years when stored at +2 to 8°C. In our research, BSA, with a molecular weight of 58 kDa, is used as the model protein to explore polymeric microsphere systems suitable for protein delivery.

## 2.11 Future Directions of Controlled Drug Delivery

Advances in nanotechnology are expected to contribute significantly to further development of drug delivery systems. In the near future, we anticipate the drug delivery industry will continue to be an important segment of the pharmaceutical industry. We expect to see more and more pharmaceutical companies competing to

explore drug delivery technologies and improved drugs. These reformulation opportunities only exist because drug delivery technologies were not harnessed before the branded products' patent expirations.

In conclusion, the market of controlled drug delivery has become a long way and will continue to grow at a significant rate. Since more and more large pharmaceutical companies are involving in the development of new controlled drug delivery technologies, tomorrow's drug will definitely be more challenging because of the development of drug delivery systems.

## 2.12 Research Objectives

Currently, POE polymers are receiving significant attention because some of them undergo surface-dominant erosion and consequently control erosion rates. POE polymers have been used to encapsulate water-soluble drugs such as 5-fluorouracil and proteins within microparticles using a double emulsion solvent evaporation/extraction technique [82, 83]. It has been proved that POE polymers provided a friendly environment for proteins. However, since POE is a highly hydrophobic polymer, the affinity between POE and the water-soluble protein is weak. This yields an unstable emulsion and leads to a poor protein encapsulation efficiency. When PEG is built into the POE chain such as POE-PEG-POE block copolymers, or blended with POE, it can be expected that the triblock copolymer and the POE/PEG blend will stabilise the protein and enhance protein encapsulation efficiency. Based on these promising polymer systems, our research focuses are summarized as follows:

1. To fabricate POE/PEG blend and POE-PEG-POE triblock copolymeric microspheres, and to investigate the morphology, particle size, protein distribution and protein encapsulation efficiency of the microspheres under different fabrication conditions.

2. To study the degradation and erosion mechanism of the microspheres. In addition, to explore the stability of an encapsulated protein and its release properties.

3. To improve protein release profiles based on the understandings of protein release and polymer erosion mechanisms.

# CHAPTER THREE

# MATERIALS AND METHODS

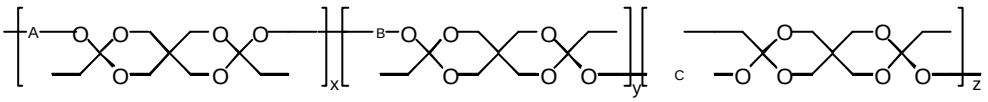
The materials and methodology used in the course of the research project are presented below. Included are the methods for microsphere preparation as well as microsphere post-fabrication analysis.

## 3.1 Materials

### 3.1.1 Polymers

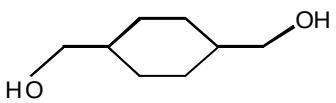
PEG polymers, with number molecular weight of 4,600, 100,000 and 200,000, were purchased from Sigma-Aldrich Company. POE and POE-PEG-POE triblock copolymers having a 4,600 or 1,000 number molecular weight of poly (ethylene glycol) (PEG) were synthesized and supplied by AP Pharma. Inc., USA. The synthesis of POE-PEG-POE was described in *Section 3.2*. The PEG content ranges from 5% to 30%. Table 3.1 lists the properties of POE-PEG-POE triblock copolymers. The chemical structures of POEs used in this study are listed in Figure 3.1.

**POE**

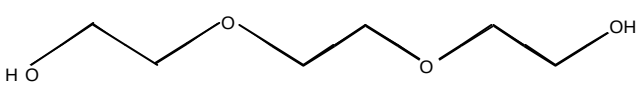



*(A/B/C: CDM/TEG/TEG-diGL; x:y:z=94:5:1; when A and B stand for 1,2-PrD/1,2-PrD-diGL, x:y=95:5, 90:10 and 85:15)*

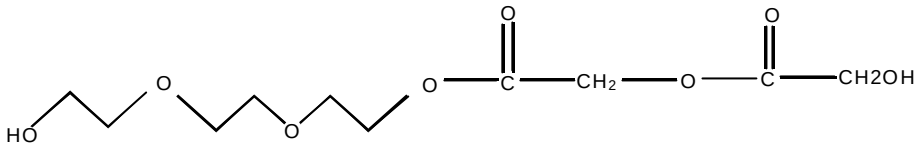
**CDM**



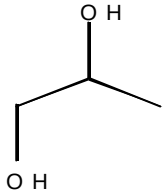

**TEG**



**TEG-diGL**



**1,2-PrD**



**1,2-PrD-diGL**

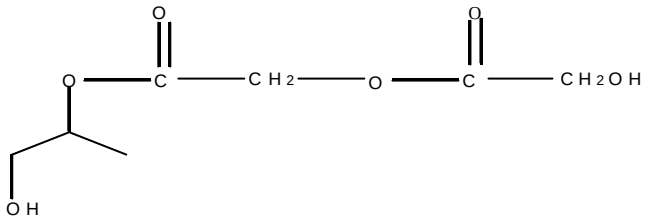


*Figure 3.1 Chemical structure of POE.*

*Table 3.1 Properties of POE-PEG-POE triblock copolymers.*

| Copolymer type | PEG Content (in weight) | Mw |
|---|---|---|
| POE | 0 | 46.0k |
| POE-PEG(5%)-POE | 5% | 41.6k |
| POE-PEG(10%)-POE | 10% | 36.9k |
| POE-PEG(20%)-POE | 20% | 23.3k |
| POE-PEG(30%)-POE | 30% | 12.3k |

### 3.1.2 Protein

Bovine serum albumin (BSA) (fraction V, 58kDa), used as the model protein, was supplied by Sigma-Aldrich Company.

### 3.1.3 Emulsifiers and solvents

Poly (vinyl alcohol) (PVA) (87-89 mol % hydrolyzed, Mw 31,000-50,000) and polyoxyethylene-sorbitan monooleate (Tween 80) were purchased from Sigma-Aldrich Company. Methylene chloride (L. C. grade) was purchased from Merck. Deuterated chloroform ($CDCl_3$) was purchased from Sigma Company. All other chemicals were of reagent grade and used as received.

## 3.2 Synthesis of POE-PEG-POE Triblock Copolymers

The synthesis of the POE-PEG-POE triblock copolymers was carried out in AP Pharma. Inc., USA, which is illustrated by the following example of POE-PEG-POE with a PEG content of 5% in weight and a number molecular weight of 4,600.

Under an anhydrous condition, the mixture of 3,9-diethylidene-2,4,8,10-tetraoxaspiro{5,5} undecane (DETUSO) (21.225g, 100 mmoles) and PEG (Average Mn = 4,600, 2g, 2 mmoles) was dissolved in 40 ml of tetrahydrofuran (THF). After addition of p-toluenesulfonic acid (PTSA)/THF solution (0.05 ml, 20 mg/ml), the reaction mixture was stirred for 30 minutes. Next, the solution of *trans*-cyclohexanedimethanol (CDM) (13.29g, 92.12 mmoles), tri (ethylene glycol) (TEG) (0.736g, 4.9 mmoles) and tri(ethylene glycol)-diglycolide (TEG-diGL) (0.216g, 0.98 mmoles) in THF (40ml) was added to the reaction mixture. After another portion of PTSA/THF solution (0.05ml, 20 mg/ml) was added, the reaction mixture became boiling. The solution was stirred until it cooled to room temperature. The polymer was precipitated from 1.5L of methanol containing 0.5 ml of triethylamine as a

stabilizer, and dried in a vacuum oven, first at 30°C overnight then at 80°C for 5 hours.

## 3.3 Polymeric Microspheres Preparation

BSA-loaded POE, POE-PEG-POE and POE/PEG blend microspheres were prepared using an improved double-emulsion (water-in-oil-in-water) solvent evaporation/extraction procedure [84]. Briefly, 600mg polymer (or polymer blend) was dissolved in 12ml methylene chloride. After that, 0.3 ml* of BSA-containing PBS solution (the internal aqueous phase) was emulsified in the polymer solution for 15 seconds by sonication to produce a primary W/O emulsion. This emulsion was injected with stirring into 250 ml of PBS solution containing PVA as an emulsifier (the external aqueous phase) to produce a W/O/W double emulsion. The solution was maintained at 15°C with stirring for 30 minutes using a mixer (Cole-Parmer Instrument Co., IL USA). Then, 640 ml of PBS containing PVA was added at a rate of 3 ml/min for about four hours. The resultant microspheres were filtered, washed and vacuum-dried overnight and stored at 4°C before use.

## 3.4 Evaluation of Protein Encapsulation Efficiency

The BSA amount lost in the external aqueous phase during the fabrication process was analyzed using a high performance size exclusion chromatography (HPSEC). The HPSEC system consisted of a 1050 Quatern Pump, a 1100 autosampler injector and a Diode-Array UV detector. A Zorbax GF-250 column (4.6 mm × 25 cm, Dupont

Company) was used as the separation column. The flow rate of the mobile phase (PBS, pH 7.0) was 1.0 ml/min and the UV detection was at 210 nm. The encapsulation efficiency was calculated as the ratio of actual and theoretical BSA content as illustrated in equation 3.1:

$$\textit{BSA Encapsulation Efficiency (EE) \%} = \frac{\textit{BSA encapsulated, mg}}{\textit{Theoretical amount of BSA fed, mg}} \times 100\% \qquad (3.1)$$

## 3.5 Determination of Inherent Viscosity ($\eta_{inh}$) of Polymer Solution

The inherent viscosity of POE, POE-PEG-POE copolymer and POE/PEG polymer blend in methylene chloride was determined according to ISO/DIS 3105 and ASTM D2515/D446 using the Schott Gerate AVS360, Viscometer: DIN Ubbelohde (Type: Capillary No. 52510/I Appt. No. 904473) at 25 °C. The inherent viscosity is calculated using the equation 3.2 as following:

$$\eta_{inh} = \frac{\ln(t/t_0)}{C} \qquad (3.2)$$

*where $t_0$ is flow time of solvent,*

*t is flow time of polymer solution and*

*C is the concentration of the sample in g/dL.*

* * If there is no further mentioning, the internal aqueous phase is always 0.3ml.

600 mg of solid polymer was dissolved in 12 ml of solvent and filtered (Whatman filter paper) before being filled into the viscometer. Sample concentration was 0.5 g/dL.

## 3.6 Optical Observation of Microsphere Shrinkage

The formation process of microspheres were tracked with a Nikon polarizing microscope (Optiphot 2-pol, Japan)

### 3.6.1 Initial formation

The first sample was drawn immediately after injection of the first emulsion into the external aqueous phase. For next 30 minutes, photomicrographs of the sample were captured continuously at 5-minute intervals. This was taken as the initial formation of microspheres.

### 3.6.2 Continuous formation

Following this, samples were continuously drawn at 30-minute intervals after the first 30 minutes throughout the whole dosing period. Photomicrographs were taken for all the samples. Microsphere size was analyzed by the built-in ImagePro software. The mean diameter of the microspheres was taken to be the number average of the diameters of the particles captured on the computer image for over 300 microspheres.

## 3.7 Particle Size Distribution

The mean diameter and particle size distribution of the dried microspheres were measured using a laser light-scattering particle size analyzer (Coulter LS 230, a

microvolume module, Coulter Corporation, USA) after soaking the microspheres in an aqueous solution containing Tween 80 (0.1 w/v %).

## 3.8 Morphological Analysis

A scanning electron microscope (SEM, Model JSM-5310, JEOL, Tokyo, Japan) was used to examine the surface and internal morphology of the microspheres. For this study, 10 mg of microspheres were embedded into a Lipshaw M1 matrix (Shandon Lipshaw Inc, Pittsburgh, USA) under liquid nitrogen and sectioned using a cryostat (Leica CM 3050, Leica Instrument Gmbh, Nussloch, Germany). The microspheres and their sectioned samples were mounted on metal holders and vacuum-coated with a gold layer prior to the examination under SEM. Thickness of the gold layer was optimized to prevent the pores on microspheres from being masked out.

## 3.9 Drug Distribution Within Microspheres

In this study, a Bio-Rad confocal laser scanning microscope (CLSM, MRC 1024, England) was used to observe BSA distribution within the microspheres using BSA fluorescence [85]. An excitation wavelength of 488 nm and a 522 DF 32 emission filter were used, and a Photo Multiplier Tube 2 (Iris: 6, Gain: 1300, low signal) was selected. The laser power was 100%. Filter blocks were T2A (560 DRLP) and B1 (Beam splitter). All the observations were conducted using the same resolution.

## 3.10 Interactions Between BSA and Polymers

A Fourier transform infrared spectrophotometer (FT-IR, Perkin Elmer Spectrum 2000) was employed to explore the interactions between BSA and various polymers using potassium bromide pellets.

## 3.11 Thermal Analysis

Glass transition temperature (Tg) was measured using a Perkin Elmer 7-series differential scanning calorimetry (DSC) (Model DSC 4, Perkin-Elmer, CT, USA) with 5-10 mg of the polymeric microspheres. The Tg was determined by first cooling the sample from 10 to –10°C and then heating to 150°C at a heating rate of 10 °C/min in a nitrogen atmosphere. All the glass transition temperatures were obtained from the second heating process.

## 3.12 Chemical Composition of the Microspheres Surface

An X-ray Photoelectron Spectroscope (XPS) was employed to investigate the chemical composition of the microsphere surfaces. The XPS measurements were performed using a VG ESCA LAB 220I-XL spectrometer with a magnesium anode source producing Mg Ka (1253.6 eV photons) X-ray with the pass energy of 20 eV for high-resolution narrow scans and 150 eV for low-resolution wide scans. The XPS data analysis was carried out using the software from VG ESCA LAB. To compensate for surface charges, all XPS binding energies were referred to the adventitious C 1s peak at the binding energy of 285.0 eV. Atomic concentration was determined from the peak areas under each peak component and correcting using the

manufacturer's atomic sensitivity factors (ASF) with an estimated maximum error of ±5%. Spectral curve fitting was performed using the manufacturer-supplied software.

## 3.13 PEG Content Remained in POE/PEG Blend Microspheres After Microspheres Fabrication

Nuclear magnetic resonance spectroscopy (NMR) was utilized to determine the actual content of PEG remaining in the microspheres after preparation. 10 mg of microspheres were placed into a glass NMR sample tube with 1 ml of deuterated chloroform solution. The proton NMR spectra were acquired on a NMR spectrometer (Bruker AC 400, Germany). The hydrogen from the methylene group of the PEG homopolymer resonated at 3.6 ppm while that of the methylene group from POE polymer appeared at 4.2 ppm. The areas under the peaks were integrated to determine the ratio of POE to PEG.

## 3.14 Water Uptake of Microspheres

After incubation in PBS (pH 7.4) for 10 days at 37 °C, microspheres were taken out and weighed immediately. The water uptake of microspheres was estimated using equation 3.3 as below:

$$\textit{Water uptake (\%)} = (W1-W2)/W2 \times 100\%, \qquad (3.3)$$

where W1 and W2 are the weights of the hydrated microspheres and dried microspheres, respectively.

## 3.15 Primary Emulsion Stability Tests

Stability of the primary emulsion (BSA/PBS droplets dispersed in the polymer/methylene chloride solution) was estimated from the de-mixing time. 0.3 ml of PBS (containing 70 mg of BSA) was emulsified in 12 ml of methylene chloride (containing 600 mg polymer blend) in an assay tube fitted with a stopper. The time required for initial macroscopic phase separation to occur was measured at room temperature (22 °C).

## 3.16 *In vitro* Release Study

*In vitro* BSA release tests of polymeric microspheres were carried out in triplicate at 37°C in PBS buffer (pH 7.4). 40 mg of the dried microspheres were suspended in 1 ml PBS buffer. *In vitro* medium from each sample was periodically removed and replaced with fresh PBS buffer. The BSA content in the *in vitro* medium was analyzed using the HPSEC system as described in *Section 3.4* and bicinchoninic acid (BCA) protein assay.

## 3.17 *In vitro* Weight Loss

170 mg of the dried microspheres were immersed in 5 ml of PBS buffer. The supernatant from each sample was removed and replaced with fresh PBS periodically parallel to the *in vitro* release tests. At preset time intervals, samples were separated and vacuum-dried to constant weight. Mass loss was examined gravimetrically.

## 3.18 Molecular Weight Distribution

Molecular weights were determined using a gel permeation chromatography (GPC) (Waters 2690, MA USA). A 10 mg amount of sample was dissolved in 5 ml of THF and the solution was then filtered. The mobile phase was THF at a flow rate of 1 ml/min. Weight and number average molecular weights were calculated from a calibration curve using a series of polystyrene standards (Polymer Laboratories Inc., MA USA, with molecular weight ranging from 1350 to 151,700).

## 3.19 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The structural integrity of BSA within the degrading microspheres was examined under non-reducing conditions using a Bio-Rad Mini-Protean III electrophoresis system [86]. Briefly, 0.5 mg of dried microsphere samples were suspended in a Tris-buffer, pH 6.8, containing 2% SDS for one hour. Then the suspensions were directly loaded into the wells with a micropipette. Electrophoresis was performed at 180 V, 100 mA. The gel was stained with 0.1% Coomassie Brilliant Blue to visualize the protein, destained with an aqueous solution of 20% ethanol and 10% acetic acid, then dried overnight.

# CHAPTER FOUR

## RESULTS AND DISCUSSION I:

## Protein-Loaded POE-PEG-POE Microspheres

The use of poly (ethylene glycol) (PEG) in controlled release systems is receiving considerable attention. PEG has several advantages such as high solubility in water and organic solvents, biocompatibility, non-toxicity, non-immunogenicity and protein resistivity [58-63]. Conjugates of proteins with PEG have proved to be able to provide prolonged protein circulation life, reduced immunogenicity and antigenicity [58, 87-88]. In particular, PEG-adenosine deaminase and PEG-asparaginase are two PEG-protein conjugates approved by FDA for the treatment of ADA deficiency and acute lymphoblastic leukaemia, respectively. In addition, PEG conjugation with anti-tumor agents is also of great interest since PEG-anti-tumor agent conjugates could improve the water-solubility and stability of such agents, thus eliminating side effects [89]. Besides, the use of diblock copolymeric micelles with hydrophilic PEG as the shell, for targeted drug delivery, has also been frequently reported [90-93].

Recently, many studies on the use of blends and copolymerization of poly(lactic acid) (PLA) or poly(lactic-co-glycolic acid) (PLGA) or poly(butylene terephthalate) with PEG for protein delivery have been undertaken [94-97]. Generally, protein release from PLA or PLGA microspheres is affected by both protein diffusion through the porous matrix, and polymer erosion. This is characterized by a high initial burst followed by a lag in release due to slow polymer degradation and an acidic environment within the microspheres matrices [98-101]. This becomes a constraint for their use in protein delivery. The introduction of PEG in the polymer backbone could increase the hydrophilicity of the polymer matrix, and create a swollen hydrogel-like environment. Thus, PLA-PEG-PLA or PLGA-PEG-PLGA copolymeric

microspheres were tailored to provide a sustained release of protein for a period of 2 to 3 weeks [101-103].

The introduction of PEG to POE polymers in our study is expected to improve protein encapsulation and its *in vitro* release pattern. In this section, the fabrication and characterization of protein-loaded POE-PEG-POE microspheres are presented, followed by the studies of erosion and protein release mechanisms of the microspheres. Finally, the optimization of protein release profiles is attempted by varying fabrication conditions and polymer compositions.

## 4.1 Fabrication And Characterization of Protein-loaded POE-PEG-POE Microspheres

In this part, I focus on the first objective of my research work, which is to prepare and characterize protein-loaded POE-PEG-POE microspheres. In particular, the effects of PEG content in the triblock copolymer, salt and emulsifier concentration in the external water phase, protein loading as well as polymer concentration on size distribution, morphology and protein encapsulation efficiency, are studied.

### 4.1.1 Effect of PEG Content

#### 4.1.1.1 Microspheres Formation Process

Microsphere formation is a very complicated chemical engineering process. Similar to the fabrication of hollow fiber membranes, the formation of microspheres can be divided into three stages: (1) droplet formation, (2) solvent removal and solidification

and (3) washing and drying (Figure 2.11). Among these stages, solvent removal and solidification process may be the dominant stage to determine morphology and release characteristics of microspheres. Jeyanthi et al. [104] reported that the dilution solvent-removal technique yielded microspheres with a uniform honeycomb-like matrix with pore sizes depending on the extent of continuous water-phase dilution. They found that the higher the extent of continuous water phase dilution (to form the second emulsion) and the higher continuous water phase addition (to form the nascent microspheres), the larger the pores.

We used POE-PEG(5%)-POE microspheres as an example to demonstrate the details of microspheres formation process (Figure 4.1). At predetermined time intervals, a certain amount of aqueous solution was taken out and observed under an optical microscope. The monitoring started 5 minutes after the first emulsion was injected to the continuous water phase. Figure 4.1 shows that the microspheres were extremely soft even after 10 minutes. At this stage, the microspheres were transparent, with small water droplets evenly distributed inside. The pores formed within the microspheres after vacuum drying are attributed to these small internal water droplets. Since BSA is water-soluble and dissolved in the internal water phase, it is believed the protein particles usually distribute around the wall of internal pores. Therefore, the distribution and the size of pores will have significant influence on the protein release. 10 min later, the nascent microspheres appeared to be black, indicating that the particles hardened because most of the solvent has been removed.

The magnified images showing the formation of internal pores are presented in Figure 4.2. The hardening process was completed within 30 minutes, suggesting that most of

the solvent might have been removed. This result is coherent with the data of particle size changes during the fabrication process, which will be reported later.

During the microspheres formation process, water was added to the double emulsion with a speed of 3 ml/min. The purpose for adding water is to facilitate the extraction of solvent and to further solidify the microspheres [104]. Properties of microspheres could be altered by varying the rate of water addition. Y. Y. Yang et al. [4, 79, 84] reported a higher water addition rate led to more rapid skin solidification, hindering the migration of BSA outwards the surface of microspheres. Thus, the protein encapsulation efficiency was increased. If the addition speed increased too fast, the encapsulation efficiency dropped. This is because at higher water addition speeds, faster mass transfer occurred. Meanwhile, the increase in the rate of water addition also caused dissolution of more drug molecules from the interior of microspheres to the continuous water phase, leading to the decrease of encapsulation efficiency [84]. Thus the water addition rate should be finely tuned. The speed of 3.0 ml/min was chosen in my research because it was found that the drug initial burst and encapsulation efficiency could be well optimized at this adding rate.

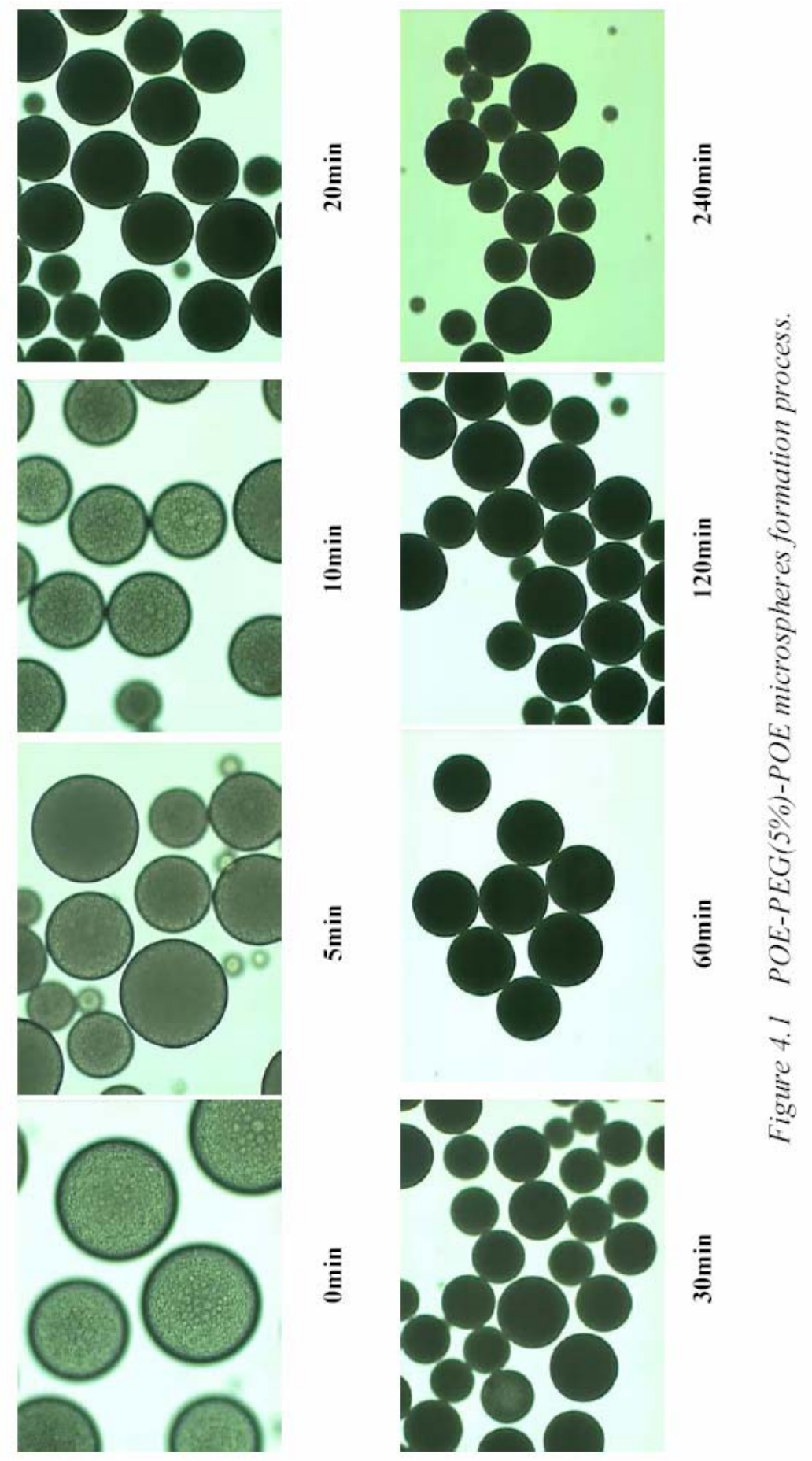



*Figure 4.1 POE-PEG(5%)-POE microspheres formation process.*

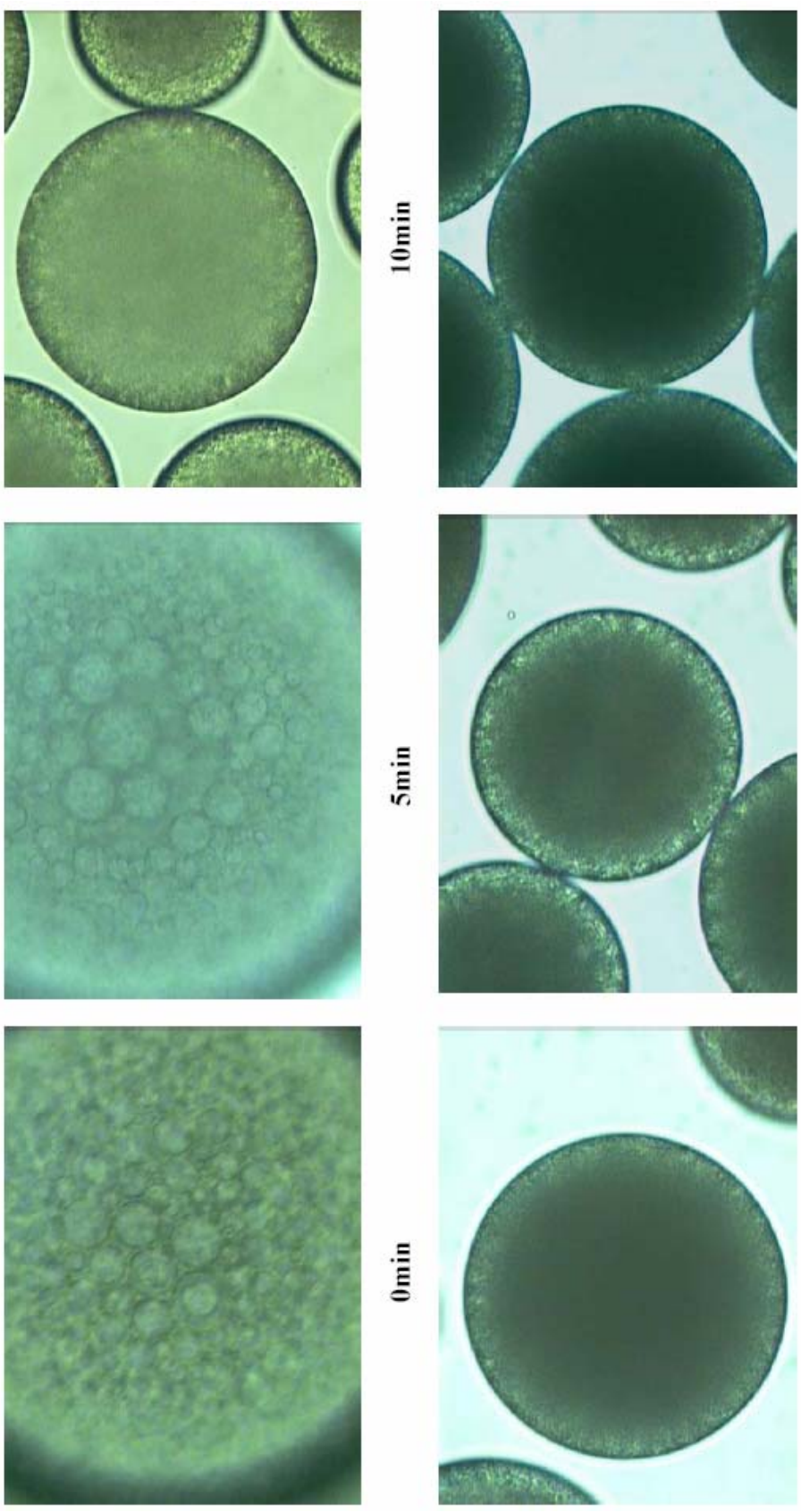



*Figure 4.2 Pore forming process during the first 30 minutes of microspheres formation. (POE-PEG(5%)-POE microspheres)*

#### 4.1.1.2 Changes in Microspheres Size During the Formation Process

The POE-PEG-POE microspheres were fabricated using the double emulsion solvent evaporation/extraction process described in Chapter 2. The microspheres shrinkage during the formation process was observed under a polarizing microscope starting at 5 minutes after the first emulsion was injected into the continuous phase. Changes in diameter of the nascent microspheres during a double emulsion fabrication process

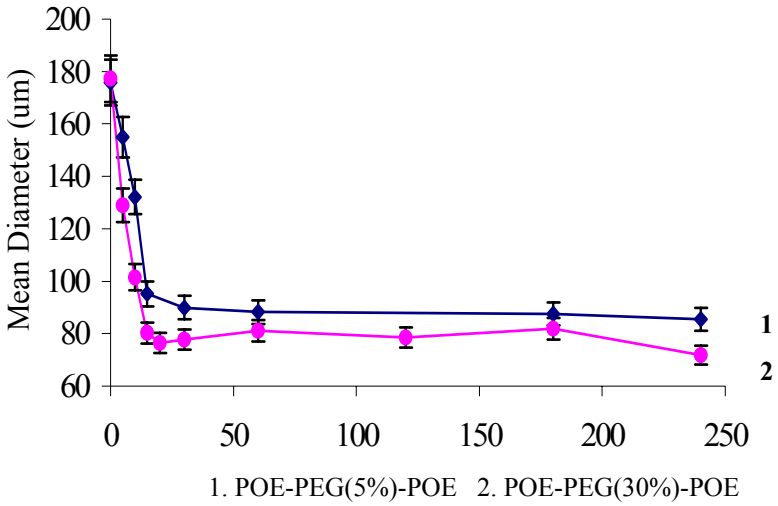



*Figure 4.3 Diameter change during the microspheres formation.*

are influenced by such factors as solvent removal, water uptake and polymer dissolution. Figure 4.3 shows typical patterns on the diameter of the microspheres made from POE-PEG(5%)-POE and POE-PEG(30%)-POE copolymers as a function of processing time. The diameter of the microspheres decreased but reached a relatively constant level after 30 minutes. On the other hand, the microspheres made from POE-PEG(5%)-POE and POE-PEG(10%)-POE under the microscope were dark and solid after 30 minutes due to removal of the solvent (methylene chloride) and fully phase inversion. However, POE-PEG(20%)-POE microspheres showed

transparent until the end of the fabrication process, resulting from swelling of the polymer that hindered microspheres solidification.

The average sizes of the microspheres made from POE-PEG(5%)-POE, POE-PEG(10%)-POE, POE-PEG(20%)-POE and POE-PEG(30%)-POE before and after vacuum drying are shown in Table 4.1, which were measured by a laser light-scattering particle size analyzer. Individual POE-PEG(30%)-POE microspheres were not obtained due to aggregation after vacuum drying. Generally, viscosity of a polymer solution has a significant effect on size of the resultant microparticles. The more viscous the polymer solution, the more difficult to be broken down into smaller droplets, leading to bigger microparticles [84]. POE-PEG(5%)-POE solution had a similar viscosity to POE-PEG(10%)-POE one (Table 4.1). Thus, the mean diameters of the resultant microspheres were quite close. However, POE-PEG(20%)-POE yielded much bigger microspheres than POE-PEG(30%)-POE even though there was no sharp difference in viscosity between these two polymer solutions. In addition, it can also be seen from Table 4.1 that the mean size of POE-PEG(20%)-POE microspheres dropped to 75.0 μm from 121.1 μm after vacuum drying but it did not change very much for POE-PEG(5%)-POE or POE-PEG(10%)-POE microspheres. This is due to the fact that POE-PEG(20%)-POE microspheres with a higher PEG content suffered more significant swelling. PEG had two effects on microspheres. The presence of hydrophilic PEG blocks in the copolymers might cause swelling of the microspheres but it might also lead to the dissolution loss of microspheres into the external water phase. Dissolution loss effect might be dominant for the POE-PEG(30%)-POE polymer. As a result, POE-PEG(30%)-POE yielded much smaller microspheres.

*Table 4.1 Sizes of POE-PEG-POE microspheres before and after vacuum drying.*

| Polymer | Inherent viscosity (dL/g) | Average size[a] (μm) before vacuum drying | | Volume diameter[a] (μm) after vacuum drying | |
|---|---|---|---|---|---|
| | | Volume mean diameter, μm | Standard deviation, μm | Volume mean diameter, μm | Standard deviation, μm |
| POE-PEG(5%)-POE | 2.48 | 91.0 | 21.4 | 80.6 | 21.3 |
| POE-PEG(10%)-POE | 2.61 | 91.7 | 24.0 | 82.0 | 18.5 |
| POE-PEG(20%)-POE | 3.25 | 121.1 | 27.3 | 75.0 | 18.7 |
| POE-PEG(30%)-POE | 3.10 | 82.3 | 24.5 | -- | -- |

[a] Measured using a laser light-scattering particle size analyser.

#### 4.1.1.3 Surface and Internal Morphology

The surface morphologies of microspheres were examined using SEM. Figure 4.4 shows that POE-PEG(5%)-POE, POE-PEG(10%)-POE and POE-PEG(20%)-POE yielded spherical microparticles. POE-PEG(30%)-POE microspheres were well dispersed while in the external water phase, but they formed aggregates after filtration and vacuum drying (Figure 4.4). In addition, SEM micrographs using high magnifications show that the surface became rougher with an increase in PEG content in the copolymers. A possible explanation is that microspheres with high PEG contents were in a highly swollen state and the skins collapsed after vacuum drying. This phenomenon was also observed in PBT-PEG and PLGA-PEG-PLGA microspheres [105, 106]. Quellec et al. reported [107] that PLA-PEG diblock copolymer yielded a PEG "brush" on the resultant nanospheres since PEG chains oriented to the external water surface during the formation process. The density of the PEG "brush" decreased with protein entrapment. The surface of POE-PEG-POE microspheres was also analyzed using XPS. Table 4.2 lists the chemical compositions of the microspheres surface. No nitrogen atoms were detected. Since nitrogen atoms were only present in BSA, this indicates that BSA was well entrapped within the microspheres matrices. Figure 4.5d shows the XPS high-resolution scans of microspheres surfaces. The largest peak at the lowest binding energy was indicative of alkane carbon (C-C) or C-H, and the smaller, intermediate peak was the C-O peak. The small, highest energy peak was due to the presence of carbon-oxygen double bond (C=O) on the surface. The peak of carbon-oxygen double bond came from POE due to the absence of BSA on the surface. In addition, from Table 4.3, we see that there was no sharp difference in the C=O content between the polymers and the

microspheres. Thus, these findings enabled us to presume that the POE-PEG-POE copolymers used in this study did not yield PEG-coated microspheres. Possibly, this was due to the spatial hindrance of triblock copolymer molecules. Also, it is observed that there was no BSA on the surface of microspheres since we could not detect the N atom in the wide scan (Figure 4.5a, 4.5b and 4.5c) while N was present in all the protein structures. The results were understandable because the hydrophilic protein should have been dissolved into the continuous water phase during the second emulsion process. The BSA amount on the microspheres surface was too little to be detected.

Figure 4.6 shows cross-section SEM micrographs of POE and POE-PEG-POE microspheres. Neat POE microspheres show a multivascular internal structure that resulted from an unstable first emulsion. POE is a highly hydrophobic polymer and the affinity between POE and BSA was weak. As a result, the first emulsion of POE/methylene chloride and BSA/PBS was extremely unstable, we observed that it separated into two phases within 1 min after sonication. During the microspheres formation process, the internal water droplets coalesced easily in a very hydrophobic environment and resulted in the multivascular structure characterised by a thick and dense wall with poor inter-pore connections (Figure 4.6 and 4.7). The attachment of PEG blocks to the POE polymer increased the polymer hydrophilicity, which stabilized the first emulsion. Thus POE-PEG-POE yielded the microspheres with a uniform pore distribution. On the other hand, from the micrographs shown in Figure 4.4, we also observe that with an increase in PEG content, the cross-section images of POE-PEG-POE microspheres show a denser internal structure. This might be due to

the fact that an increased PEG content allowed fine dispersion of internal water in the polymer matrix, which resulted in an apparently denser structure after drying.

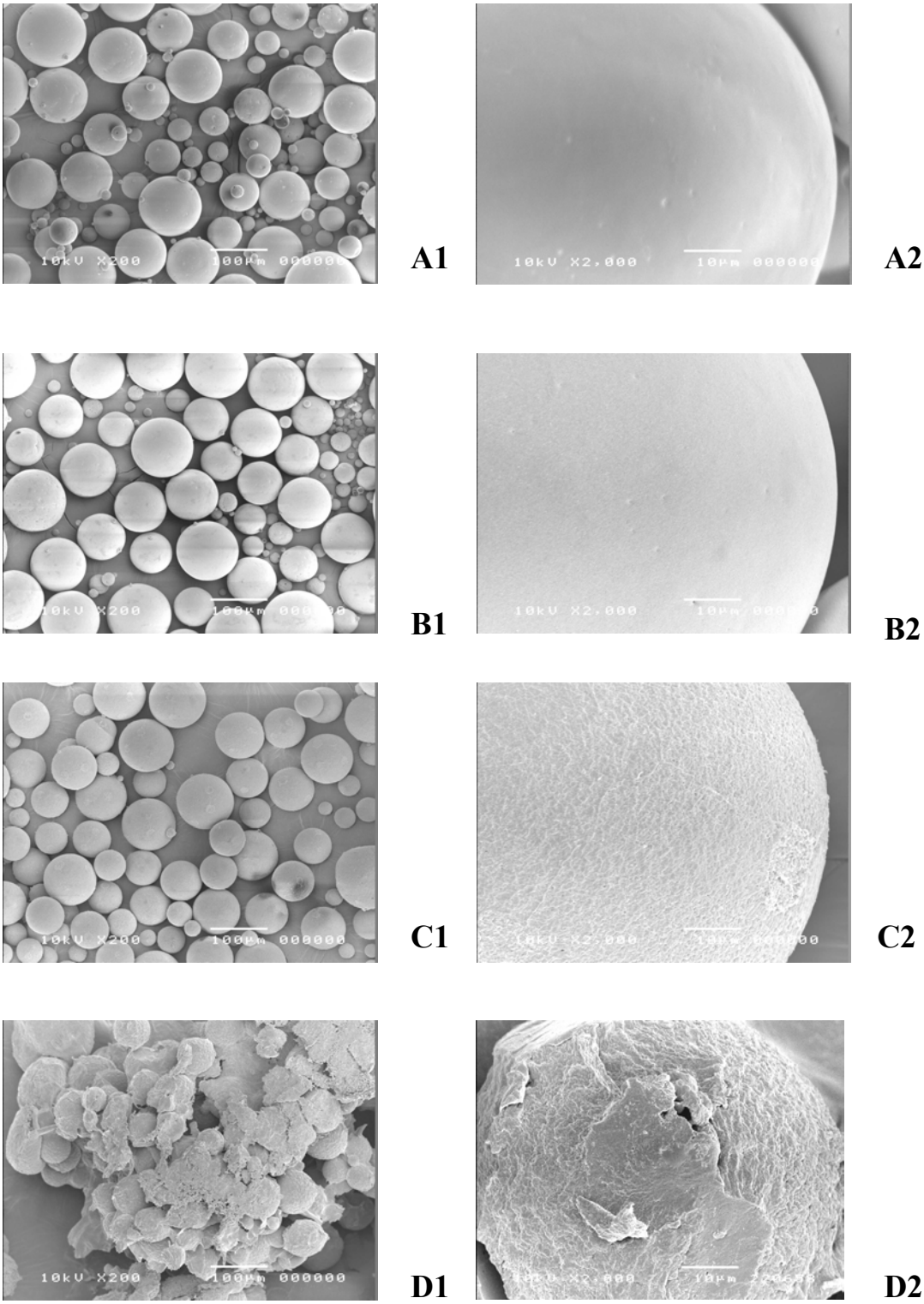



*Figure 4.4 Surface SEM scans of POE-PEG-POE microspheres with different PEG contents. A1, B1, C1, D1 represents 5%, 10%, 20% and 30%, respectively. Size of the bar is 100 μm. For A2, B2, C2 and D2, size of the bar is 10 μm.*

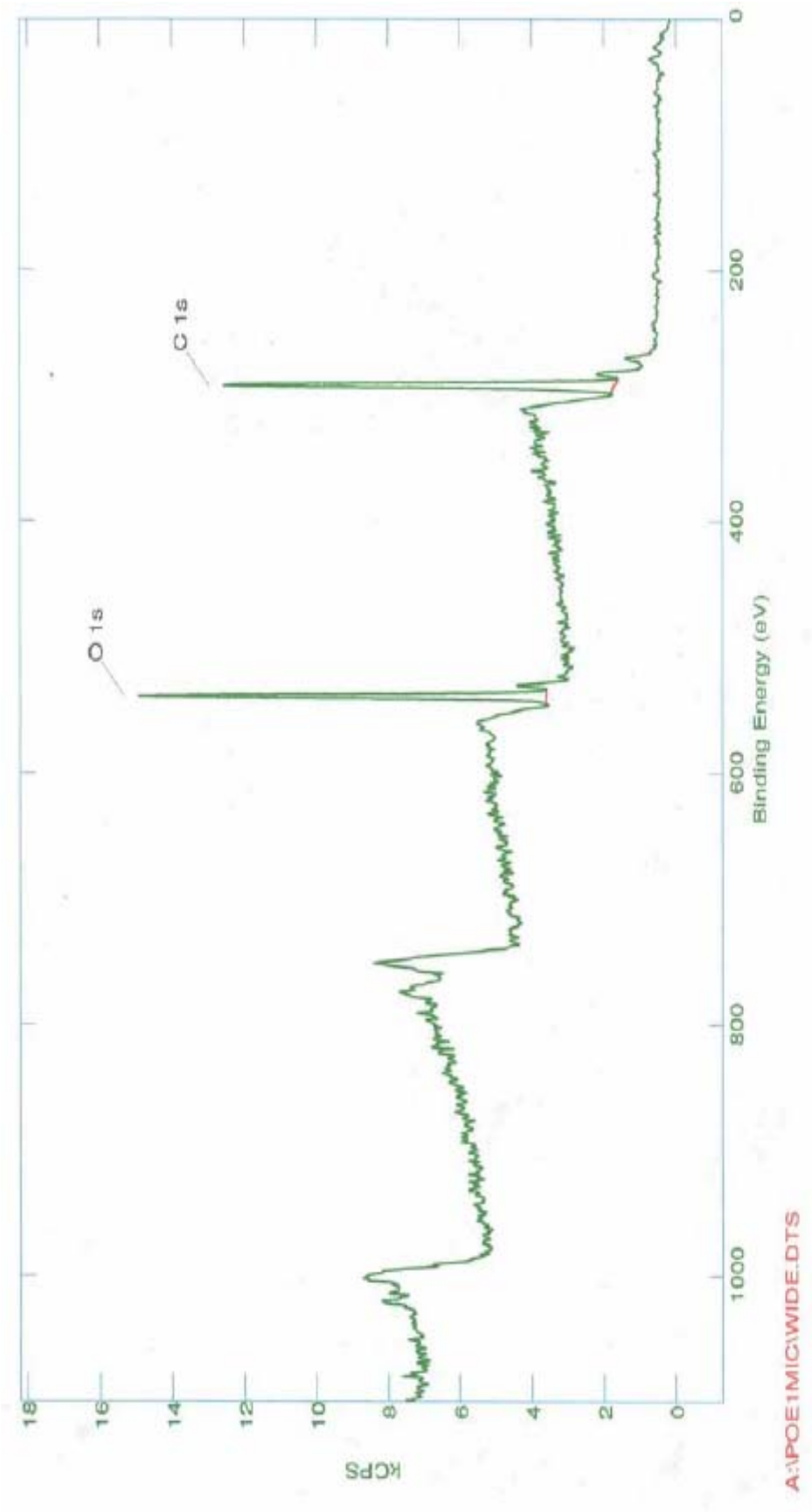



*Figure 4.5a XPS wide scan for BSA-loaded POE-PEG(5%)-POE microspheres.*

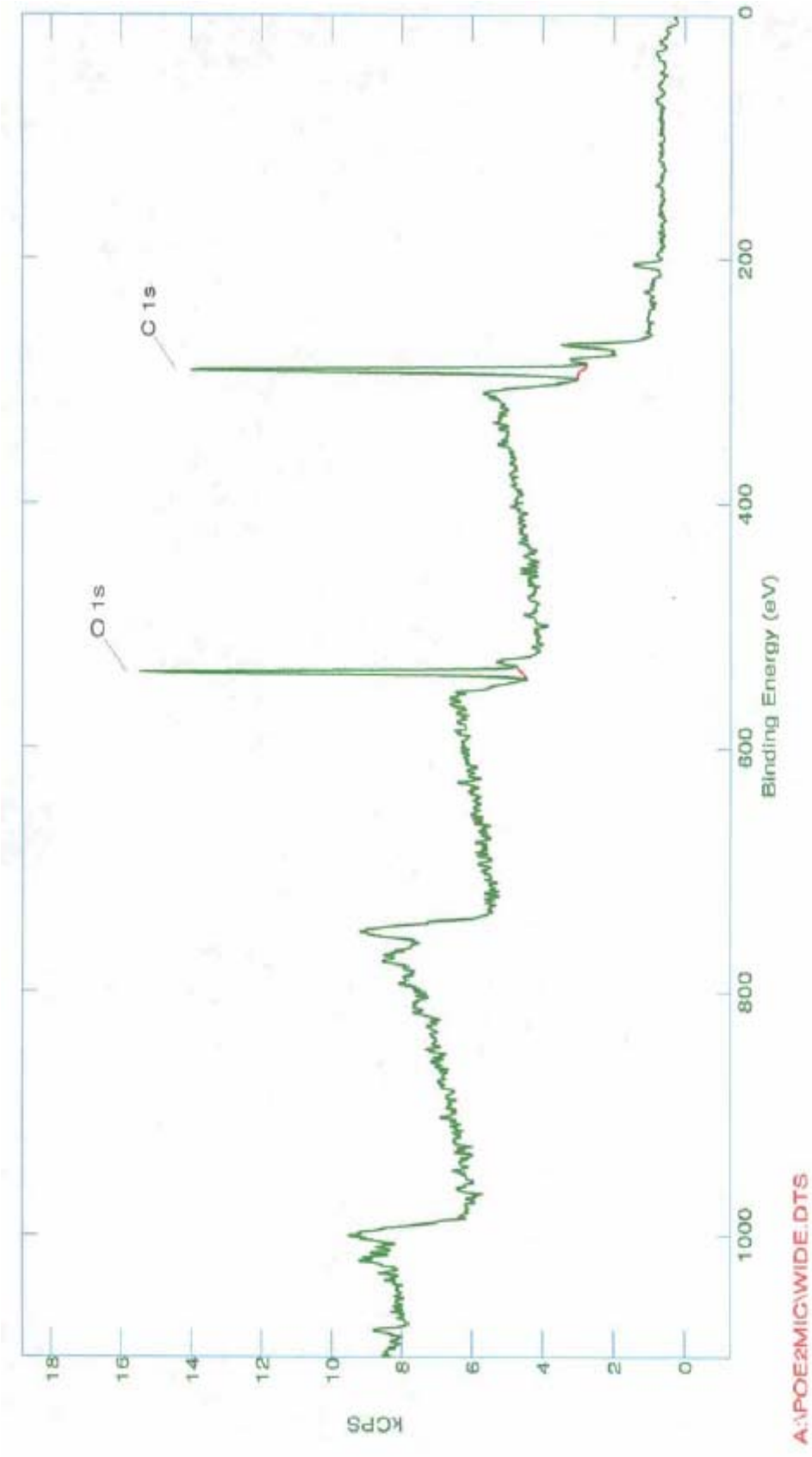



*Figure 4.5b XPS wide scan for BSA-loaded POE-PEG(10%)-POE microspheres.*

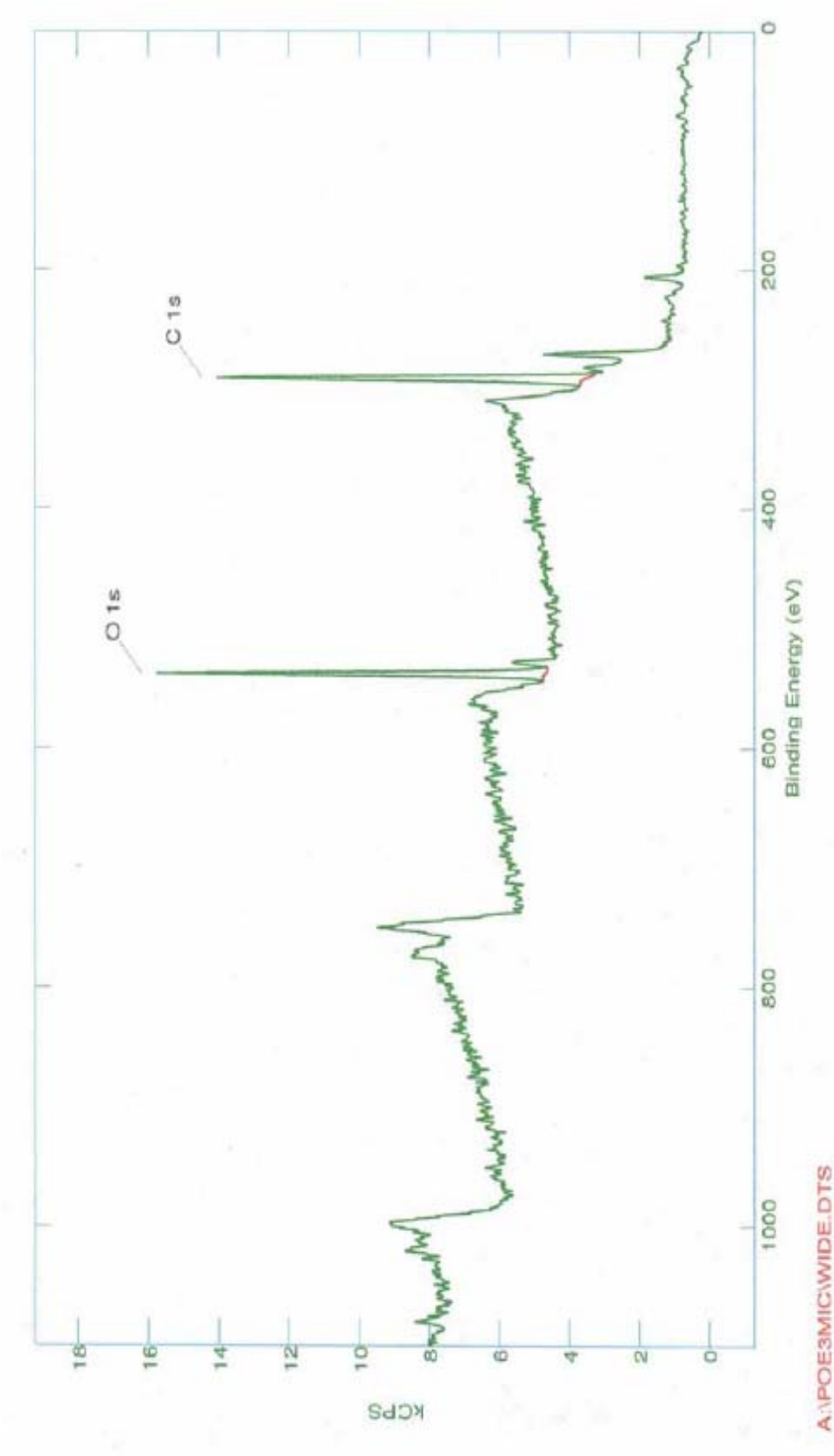



*Figure 4.5c XPS wide scan for BSA-loaded POE-PEG(20%)-POE microspheres.*

*Table 4.2 Atomic ratio of carbon and oxygen on the surface of samples.*

| Samples | C:O:N |
|---|---|
| POE | 3.05:1:0 |
| PEG | 2.11:1:0 |
| POE-PEG(5%)-POE copolymer | 2.86:1:0 |
| POE-PEG(10%)-POE copolymer | 3.00:1:0 |
| POE-PEG(20%)-POE copolymer | 2.99:1:0 |
| POE-PEG(5%)-POE microspheres | 2.86:1:0 |
| POE-PEG(10%)-POE microspheres | 2.92:1:0 |
| POE-PEG(20%)-POE microspheres | 2.65:1:0 |

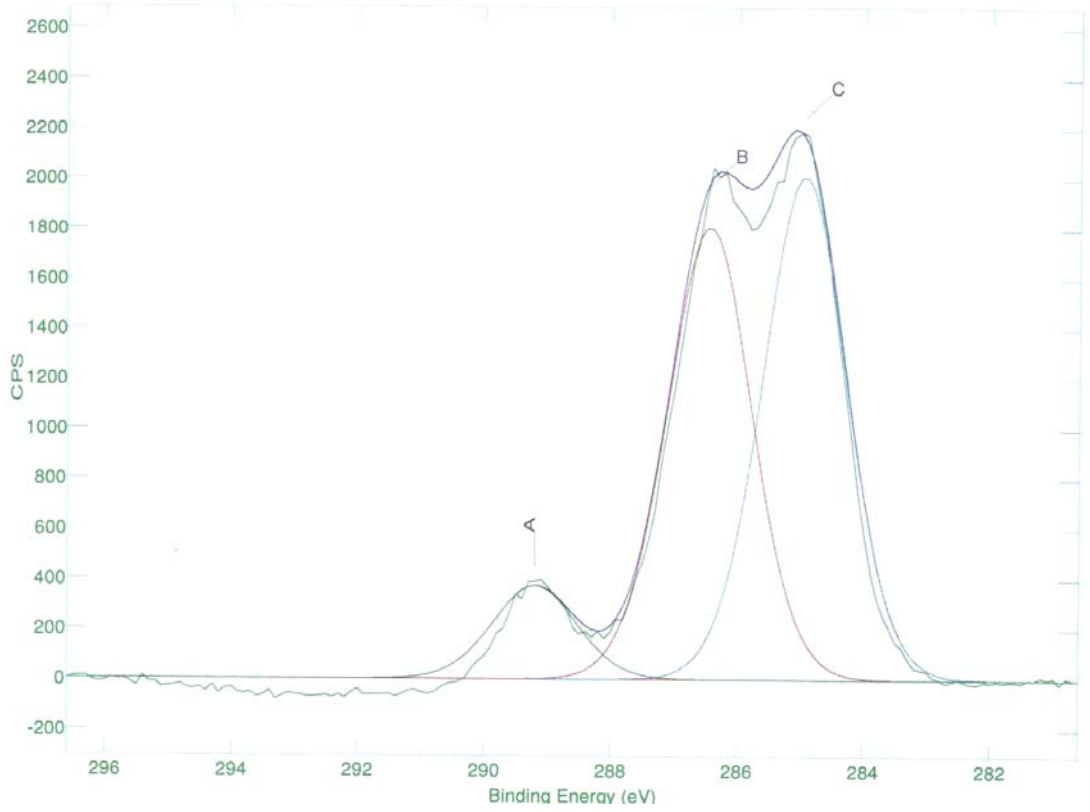



*Figure 4.5d XPS C1S high-resolution scans of POE-PEG(20%)-POE microspheres surfaces.*

*Table 4.3 Content of C=O in all the carbon bonds on the surface of samples.*

| Samples | C=O/C-O/C-C | C=O % |
|---|---|---|
| POE-PEG(5%)-POE Polymer | 1 : 3.81 : 6.81 | 8.60% |
| POE-PEG(5%)-POE Microspheres | 1 : 3.92 : 6.03 | 9.13% |
| POE-PEG(10%)-POE Polymer | 1 : 4.89 : 5.15 | 9.06% |
| POE-PEG(10%)-POE Microspheres | 1 : 4.77 : 5.74 | 8.69% |
| POE-PEG(20%)-POE Polymer | 1 : 4.59 : 5.06 | 9.31% |
| POE-PEG(20%)-POE Microspheres | 1 : 4.33 : 4.39 | 8.91% |

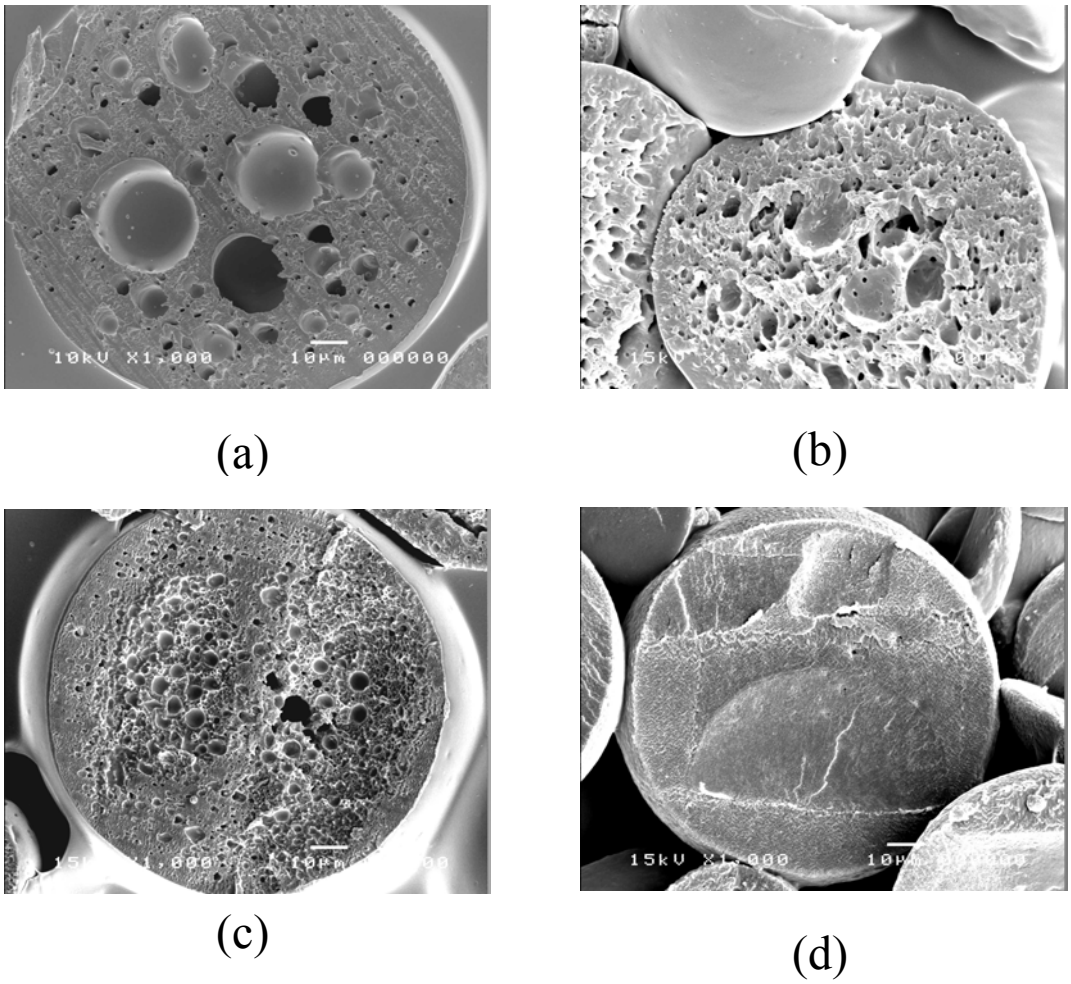



*Figure 4.6 Cross-sectional SEM scans of POE-PEG-POE microspheres with different PEG contents. (a) POE (b) POE-PEG(5%)-POE (c) POE-PEG(10%)-POE (d) POE-PEG(20%)-POE. Size of the bar is 10 μm.*

#### 4.1.1.4 Encapsulation Efficiency

A double emulsion process is the most common method used to encapsulate hydrophilic drugs especially proteins. The encapsulation efficiency of a drug depends on its solubility in water, the affinity between the drug and polymer, and the microspheres' formation process. As listed in Table 4.4, neat POE yielded 31.7% of BSA encapsulation efficiency. In sharp contrast, when PEG blocks were incorporated

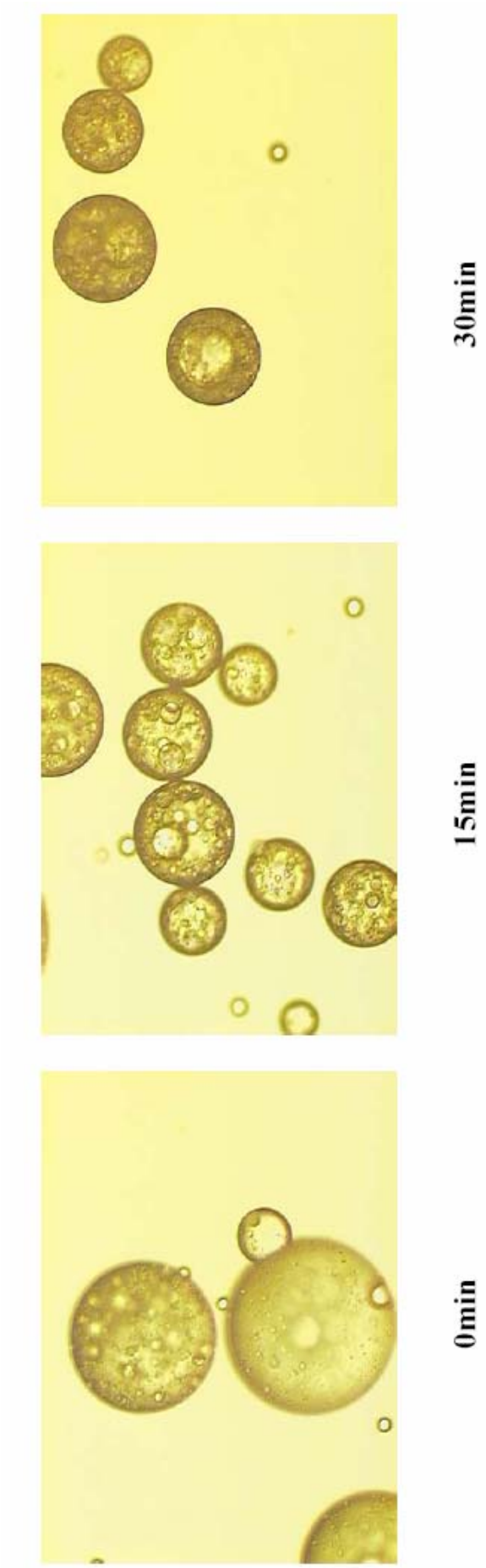



*Figure 4.7 POE microspheres formation process.*

into the POE polymer chains, an encapsulation efficiency of up to 94% was achieved. There were two possible reasons. First, the affinity between BSA and the polymer increased due to the presence of the hydrophilic PEG blocks in the polymer chain. Second, as discussed previously, POE-PEG-POE provided a more stable first emulsion so that BSA was well entrapped and dispersed within the resultant microspheres. We did the demixing tests to observe the stability of the primary emulsion. The data were presented in Figure 4.8. Comparing to copolymeric microspheres, the demixing time of primary emulsion for pure POE microspheres was much faster. It suggested the more stable first emulsion of copolymers. Interestingly, we also observed there was little difference in the demixing time among the four first emulsions. 5% PEG might be enough to stabilize the first emulsion. The results are consistent with those of BSA encapsulation efficiency. The encapsulation efficiency of BSA within POE microspheres should be closely related to the stability of the first emulsion.

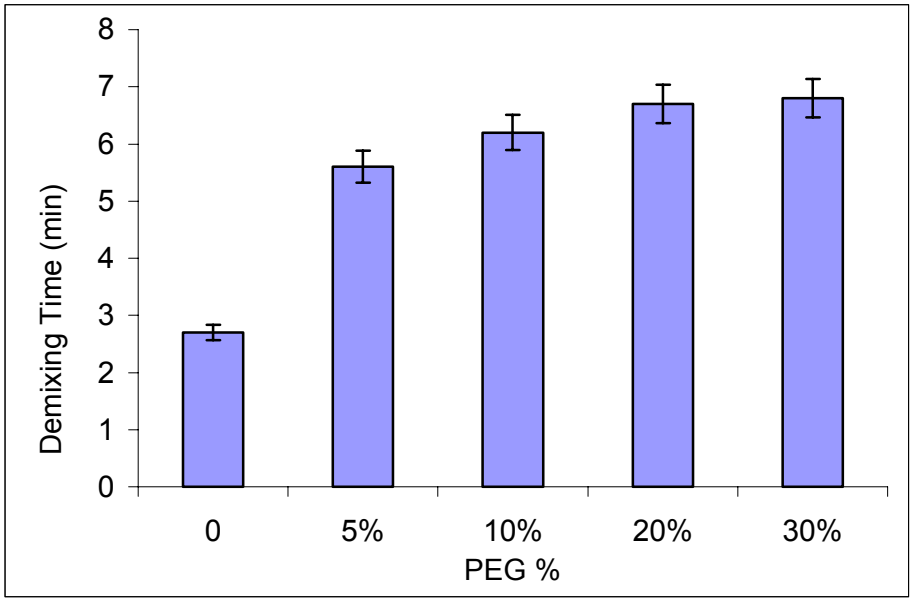
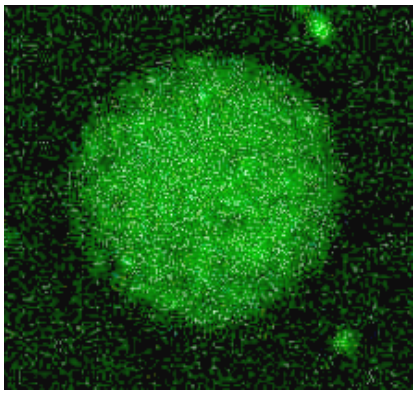



*Figure 4.8 First emulsion demixing time of POE and POE-PEG-POE microspheres with various PEG contents.*

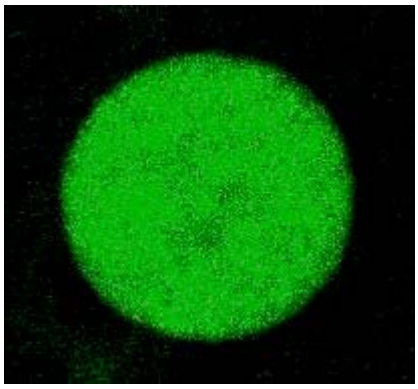
The CLSM images shown in Figure 4.9, where bright colour represented BSA, further confirm that POE-PEG-POE triblock copolymers yielded uniform BSA distribution. In this study, we attempted to use FTIR to investigate the interactions between BSA and the triblock copolymers. Figure 4.10 shows the infrared spectra of BSA, POE-PEG(20%)-POE and BSA-loaded POE-PEG(20%)-POE microspheres. The spectrum of POE-PEG(20%)-POE (Figure 4.10a) shows a carbonyl band at 1741 $cm^{-1}$ and an amide carbonyl band from BSA appears at 1654 $cm^{-1}$ (Figure 4.10b). From the spectrum of the BSA-loaded microspheres (Figure 4.10c), we observe that there are no significant shifts in these two bands. The same phenomena were observed with POE-PEG(5%)-POE and POE-PEG(10%)-POE. The results suggested the absence of strong chemical interactions between BSA and POE and the physico-chemical integrity of BSA was retained within the microspheres.

*Table 4.4 Effect of PEG content on BSA encapsulation efficiency.*

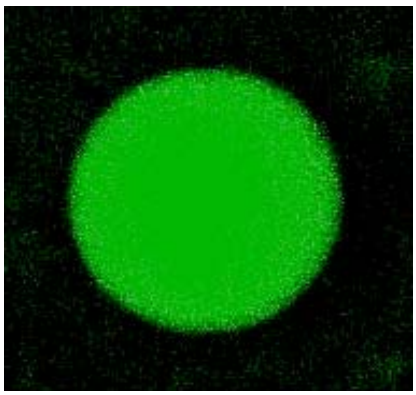
| | Initial Loading of BSA | Encapsulation Efficiency % |
|---|---|---|
| POE | 10% | 31.7 |
| POE-PEG(5%)-POE | 10% | 91.9 |
| POE-PEG(10%)-POE | 10% | 94.2 |
| POE-PEG(20%)-POE | 10% | 91.6 |
| POE-PEG(30%)-POE | 10% | 90.5 |



**(a)**



**(b)**



**(c)**

*Figure 4.9 CLSM images of POE-PEG-POE microspheres with different PEG contents. (a) POE-PEG(5%)-POE, (b) POE-PEG(10%)-POE, (c) POE-PEG(20%)-POE.*

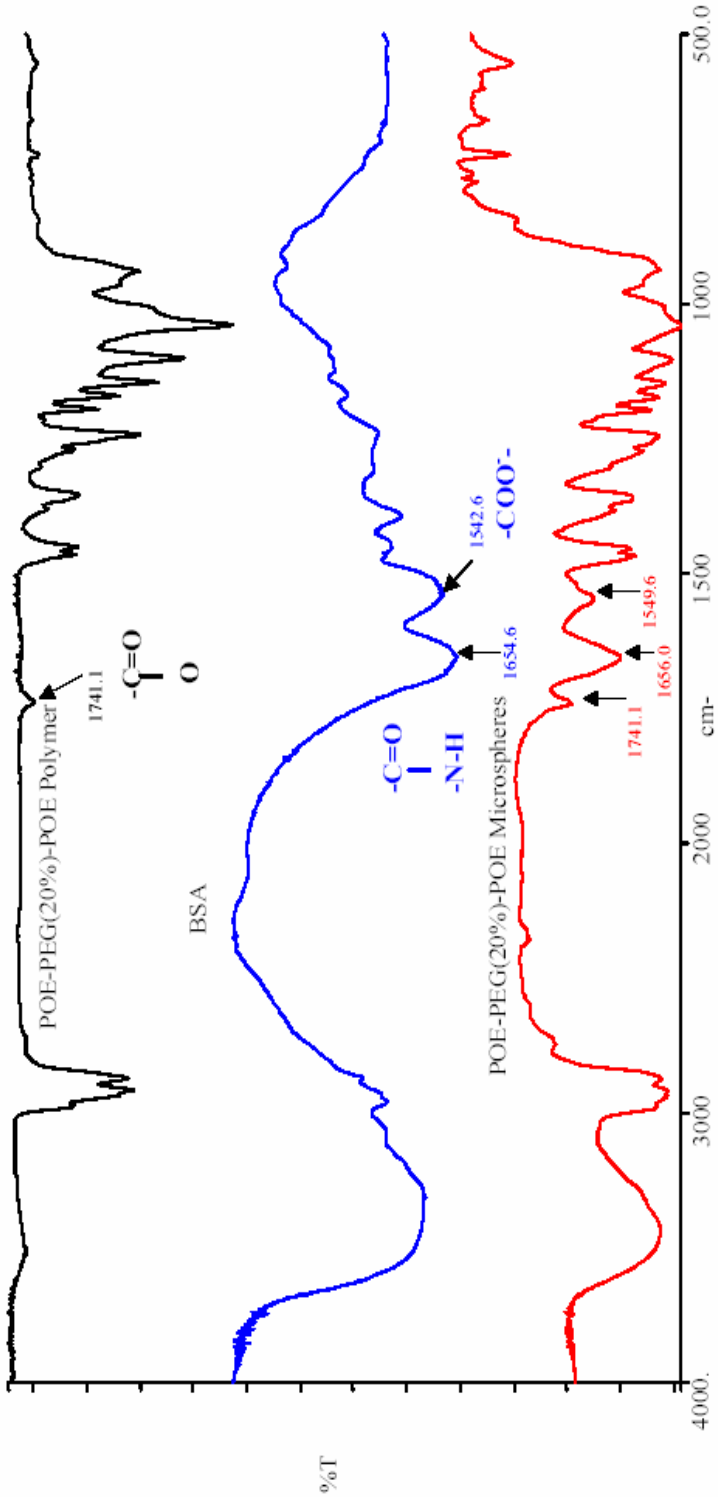



*Figure 4.10 FT-IR spectra of POE-PEG(20%)-POE copolymer, BSA and BSA-loaded microspheres.*

### 4.1.2 Effect of Salt Concentration in the External Water Phase

Thompson *et. al.* reported the encapsulation of porcine rismorelin, a highly water-soluble hormone, within PLGA microspheres using PBS as the external water phase [108]. It was also suggested that the encapsulation efficiency of an outer membrane protein (OMP) within PLA-PEG microspheres could be increased from 48.2% to 65.4% when 20% NaCl was added to the external water phase [109]. In this study, we used PBS, diluted PBS (half of its original concentration with DI water), and DI water as the external water phases to investigate salt effects. Interestingly, unlike the previous work, DI water yielded slightly higher BSA encapsulation efficiency (93.4%) when compared to encapsulations using salt (91.9%, 88.1% for PBS and the diluted PBS, respectively). This was probably attributed to the morphology of the resultant microspheres. The surface morphology was not affected by the external water phases (Figure 4.11). However, from the cross-section SEM images shown in Figure 4.11, we observe that the microspheres with dense walls were produced when half-diluted PBS and DI water were employed as the external water phase. This might be a consequence of a faster solvent removal in DI water and water solution with low salt concentrations. Actually, the presence of salt reduced the solubility of methylene chloride in water. As a result, the polymer precipitated more rapidly in water phase with the low salt concentration or DI water than in PBS solutions. As shown previously, nascent microspheres hardened during the first 30 minutes in PBS (Figure 4.2), whereas they were still transparent at 60 minutes in DI water as shown in Figure 4.12. Apparently, the polymer precipitated in DI water and half-diluted PBS formed tighter and denser skins, which hindered the exchange of methylene chloride with the surrounding medium.

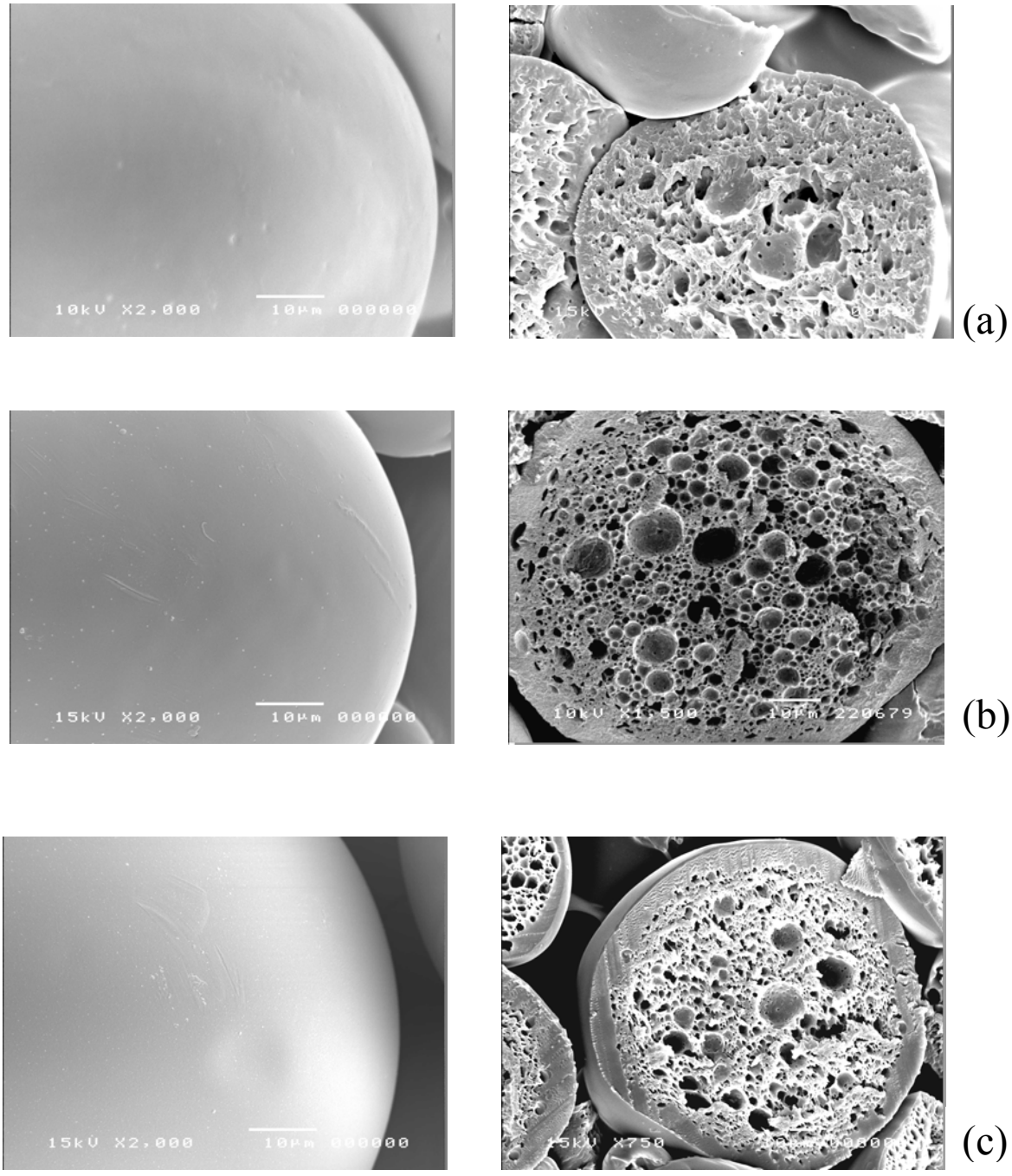



*Figure 4.11 Surface and cross-sectional SEM scans of POE-PEG(5%)-POE microspheres using (a) PBS (b) Half-diluted PBS (c) DI water as the external aqueous phase. Size of the bar is 10 µm.*

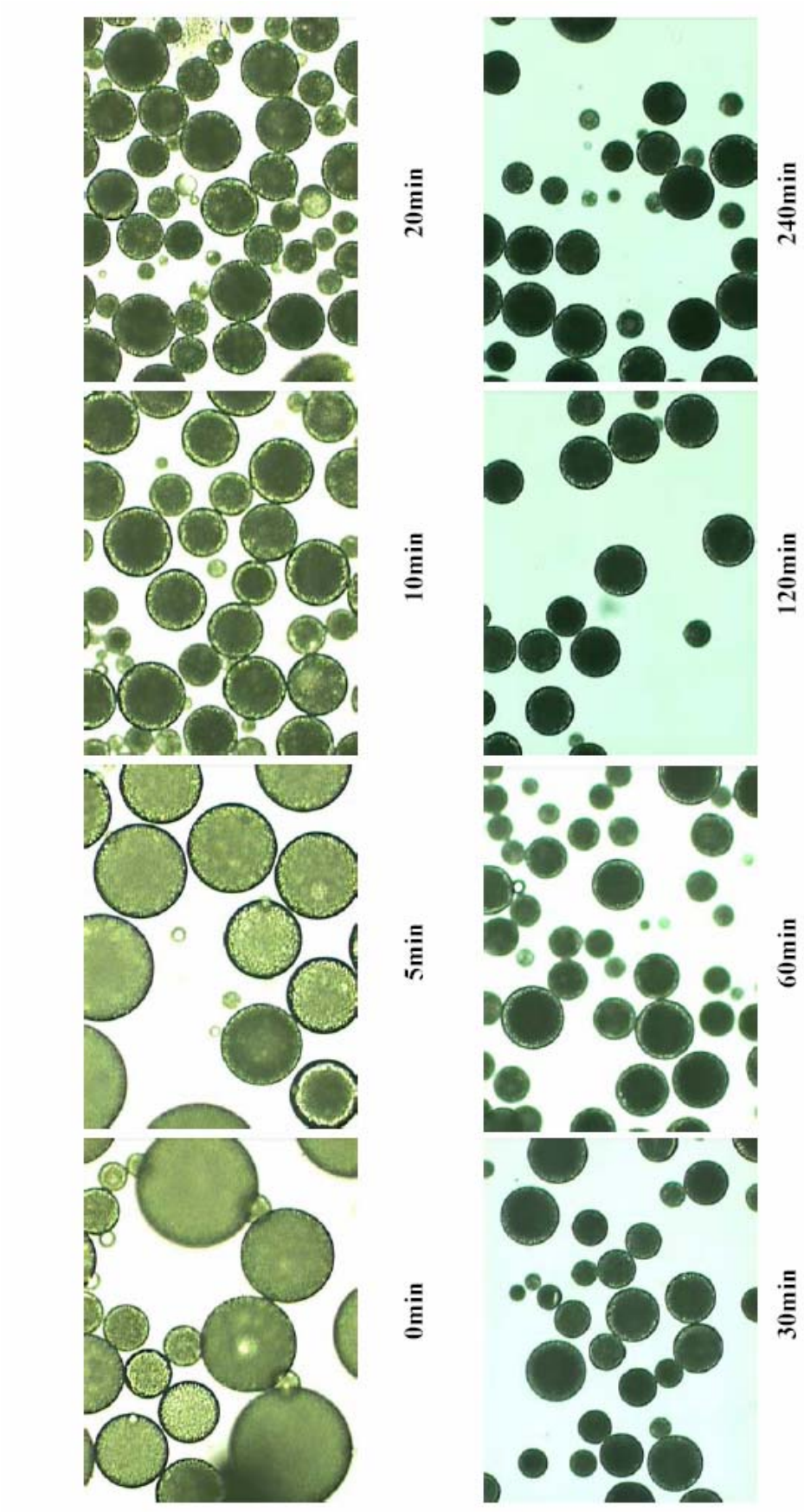



*Figure 4.12 Optical micrographs of POE-PEG(5%)-POE microspheres at various stages of solvent removal using DI water as the external aqueous phases.*

### 4.1.3 Effect of Drug Loading

To investigate the effect of BSA loading on properties of microspheres, loading was varied from 5%, 15% to 20%. Figure 4.13a shows size distribution of the resultant microspheres. An increased BSA loading led to larger microspheres because it was more difficult for the more viscous first emulsion to break down into smaller droplets. For microspheres with a thick wall, similar results were observed (Figure 4.13b). From Table 4.5, there was no sharp difference in BSA encapsulation efficiency. This was due to the stable first emulsion and the good affinity between BSA and POE-PEG(5%)-POE that prevented BSA from loss to the external water phase during the fabrication process.

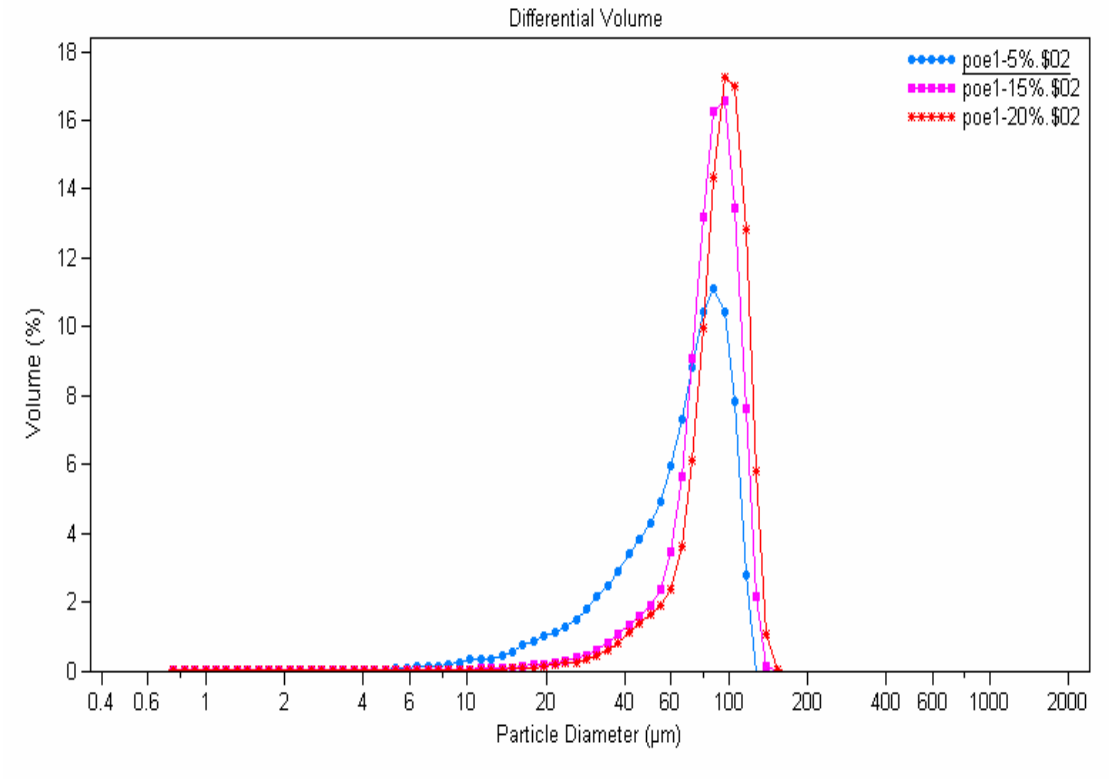



*Figure 4.13a Particle size distribution of POE-PEG(5%)-POE microspheres with 5%, 15% and 20% of BSA loadings. The mean diameter is expressed as volume diameter.*

*Table 4.5a Effect of BSA loading on properties of POE-PEG(5%)-POE microspheres.(PBS as external wter phase)*

| Initial loading of BSA | Mean diameter[a] (μm) | | Encapsulation efficiency % |
|---|---|---|---|
| | Volume mean diameter, μm | Standard deviation, μm | |
| 5% | 67.6 | 27.2 | 92.1 |
| 15% | 84.3 | 23.2 | 91.4 |
| 20% | 91.3 | 23.8 | 90.1 |

[a] Measured using a laser light-scattering particle size analyser.

*Table 4.5b Effect of BSA loading on properties of POE-PEG(5%)-POE microspheres (DI water as external water phase).*

| Initial loading of BSA | Mean diameter[a] (μm) | | Encapsulation efficiency % |
|---|---|---|---|
| | Volume mean diameter, μm | Standard deviation, μm | |
| 5% | 70.2 | 21.3 | 89.8 |
| 10% | 75.5 | 20..2 | 93.4 |
| 20% | 83.1 | 25.1 | 91.5 |

[a] Measured using a laser light-scattering particle size analyser.

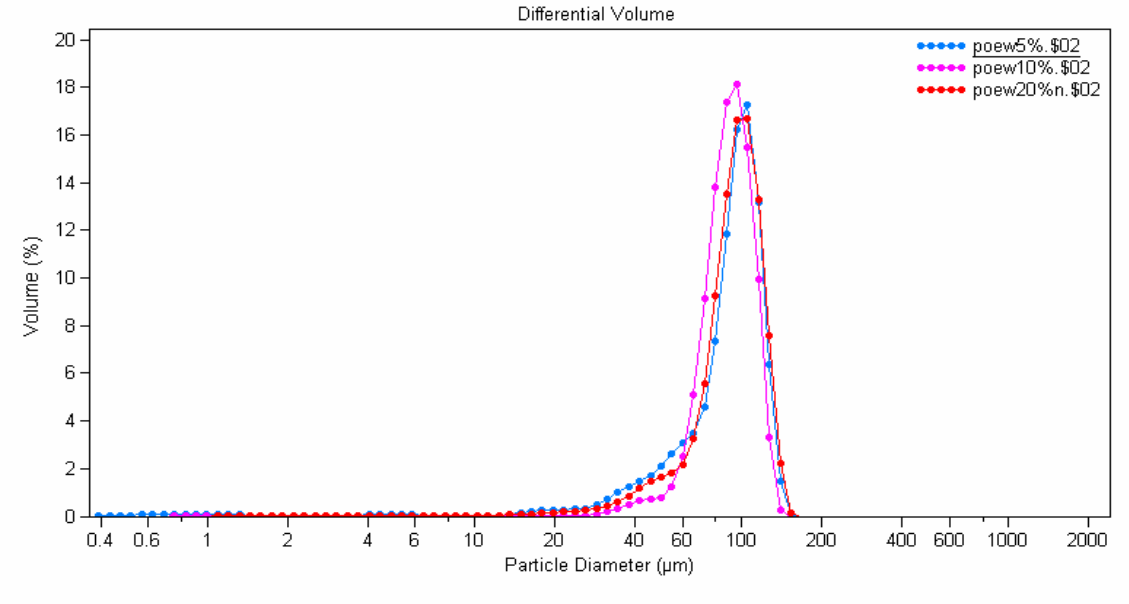



*Figure 4.13b Particle size distribution of POE-PEG(5%)-POE microspheres with 5%, 10% and 20% of BSA loadings. The mean diameter is expressed as volume diameter. (DI water as the external aqueous phase)*

### 4.1.4 Effect of PVA Concentration in the External Aqueous Phase

It is well known that particle size can be controlled by varying the emulsifier concentration in the external water phase [110, 111]. As shown in Figure 4.14, an increased PVA concentration led to a slight decrease in particle size. PVA concentration had an offsetting effect on size of microspheres. Since PVA is a polymer with a high molecular weight, the presence of PVA in the external water phase might increase the viscosity of the double emulsion, resulting in an increased difficulty in breaking up the emulsion into smaller droplets. Thus, this yielded bigger microspheres. On the other hand, the presence of PVA in the external water phase stabilized emulsion droplets against coalescence, resulting in smaller emulsion droplets. In our present work, it can be concluded that the stabilization effect was dominant at higher PVA concentrations and led to the decrease in the size of

microspheres. Table 4.6 indicates that there was no considerable effect on the BSA encapsulation efficiency because of the good affinity between BSA and POE-PEG-POE copolymers as discussed previously. In addition, there was no obvious difference in terms of surface and internal morphologies of microspheres fabricated with different PVA concentrations (Figure 4.15).

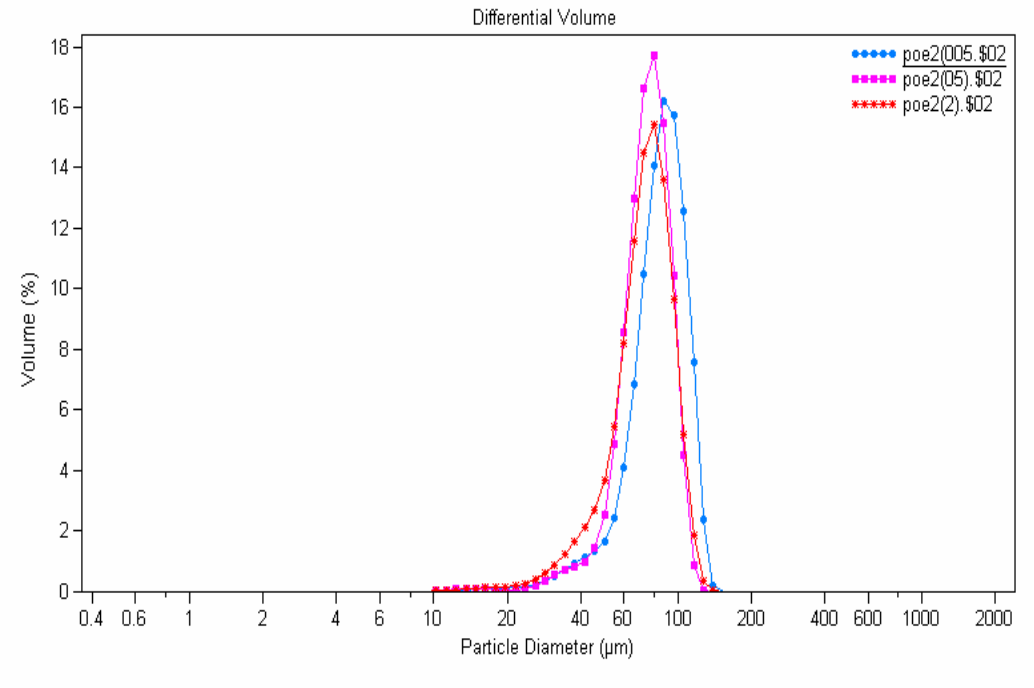



*Figure 4.14 Particle size distribution of POE-PEG(10%)-POE microspheres with 0.05%, 0.5% or 2.0% of PVA concentration in the external water phase.*

*Table 4.6 Effect of PVA concentration in the external water phase on properties of POE-PEG(10%)-POE microspheres.*

| PVA concentration (w/v %) | Mean diameter[a] (μm) | | Encapsulation efficiency % |
|---|---|---|---|
| | Volume mean diameter, μm | Standard deviation, μm | |
| 0.05 | 85.0 | 21.2 | 95.3 |
| 0.5 | 75.5 | 16.8 | 96.0 |
| 2.0 | 73.7 | 19.8 | 93.7 |

[a] Measured using a laser light-scattering particle size analyser.

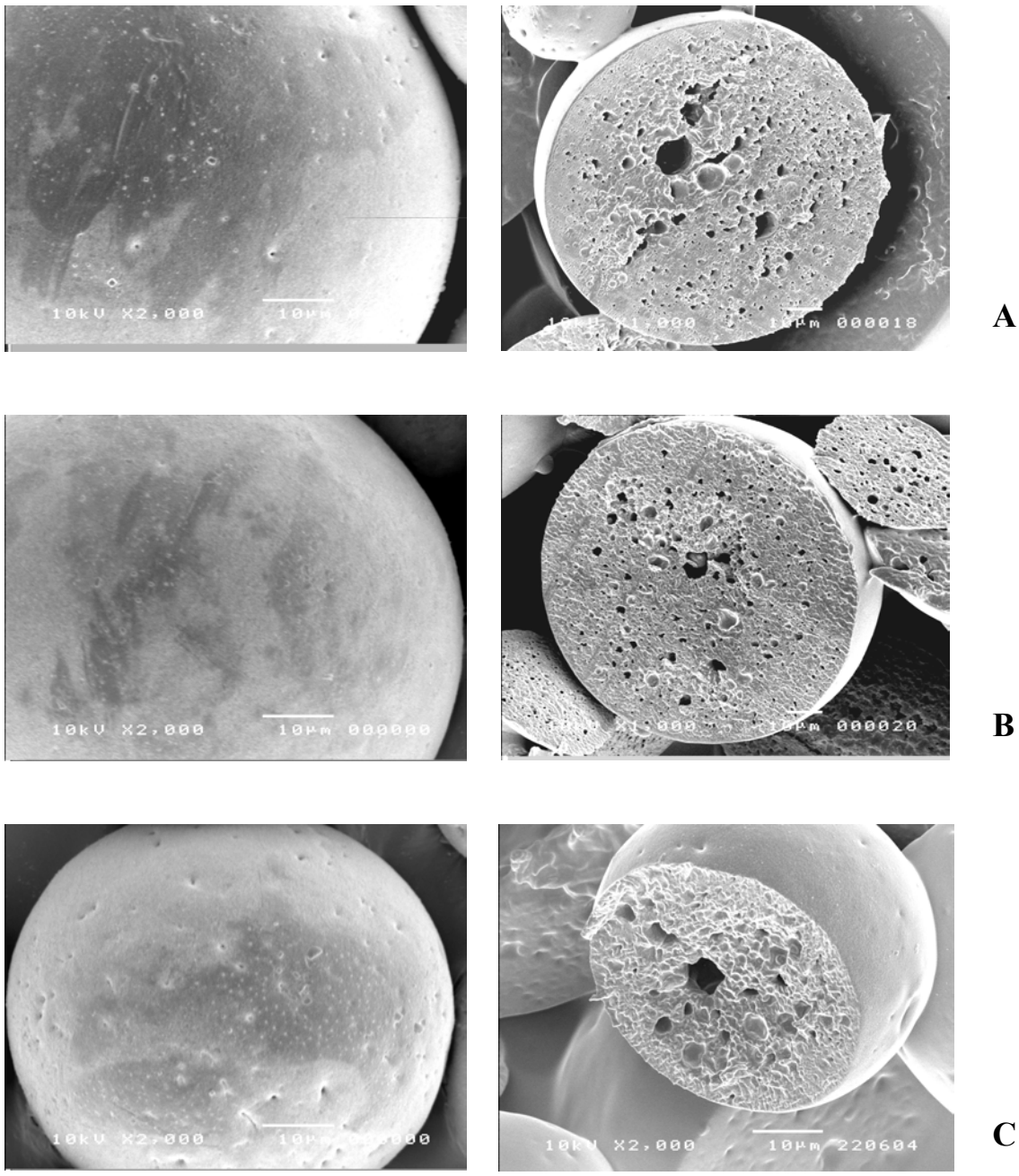



*Figure 4.15 Surface and cross-sectional SEM scans of POE-PEG(10%)-POE microspheres using different emulsifier concentrations in the external water phase. A, B, C represents 0.05%, 0.5% and 2.0% PVA, respectively. Size of the bar is 10 um.*

### 4.1.5 Effect of Polymer Concentration

Figure 4.16 shows particle size distribution of microspheres fabricated with various polymer concentrations. Clearly, the polymer concentration played a greater impact on particle size than the PVA concentration described above. When the polymer concentration decreased from 50.0 mg/ml to 16.7 mg/ml, the average size of POE-PEG(10%)-POE microspheres dropped to 53.2 μm from 76.5 μm due to the decrease in viscosity of polymer solution. As shown in Table 4.7, the polymer concentration did not affect the encapsulation efficiency much owing to the good affinity between BSA and the polymer. However, from Figure 4.17, a reduced polymer concentration produced microspheres with more porous surface and internal structures. This might be due to two factors: (1) the internal water droplets in the low polymer concentration solution tended to coalesce together more easily, leading to bigger pores and a less tortuous network, (2) the higher polymer concentration solution coagulates faster during the second emulsion and yielded a tighter structure because of chain entanglement.

*Table 4.7 Effect of polymer concentration on properties of POE-PEG(10%)-POE microspheres.*

| Polymer concentration (mg/ml) | Mean diameter[a] (μm) | | Encapsulation efficiency % |
|---|---|---|---|
| | Volume mean diameter, μm | Standard deviation, μm | |
| 16.7 | 53.2 | 14.3 | 96.1 |
| 33.3 | 71.0 | 18.7 | 95.7 |
| 50.0 | 76.5 | 19.7 | 94.2 |

[a] Measured using a laser light-scattering particle size analyser.

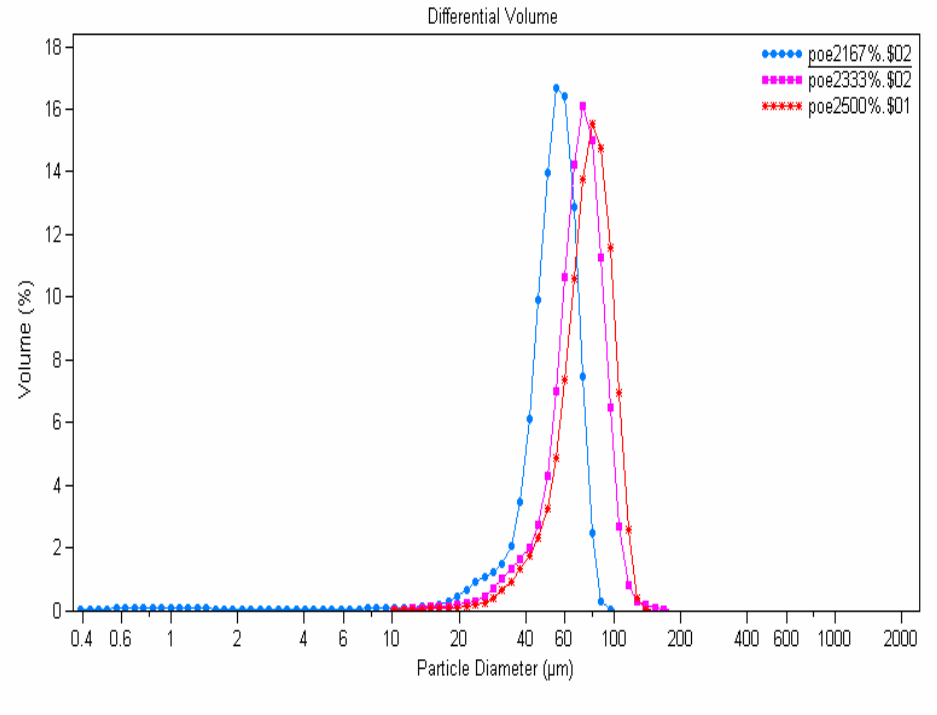



*Figure 4.16 Particle size distribution of POE-PEG(10%)-POE microspheres with 16.7, 33.3 or 50.0mg/ml of polymer concentration.*

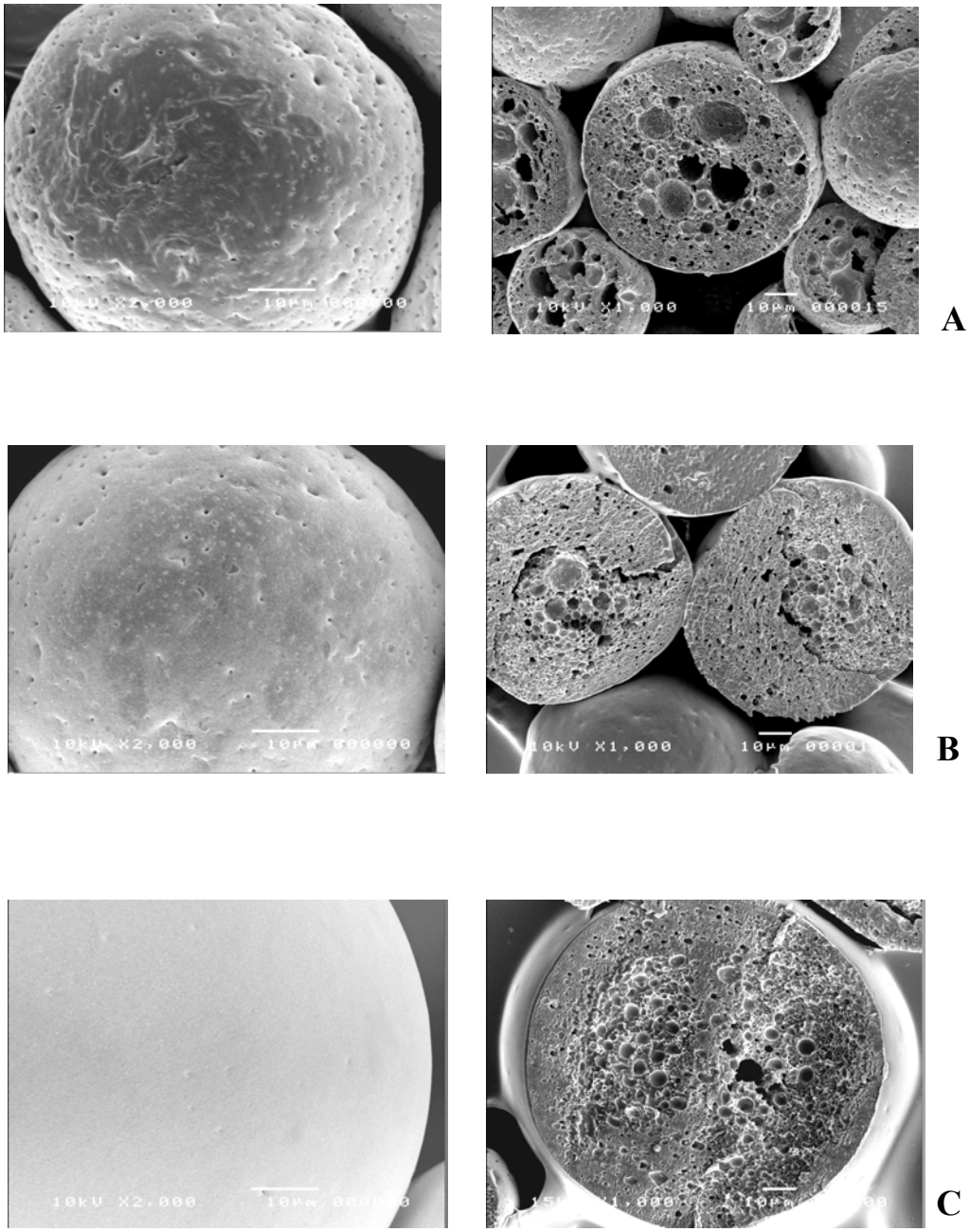


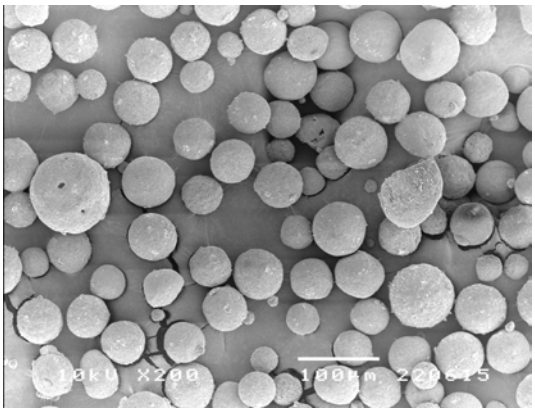

*Figure 4.17 Surface and cross-sectional SEM scans of POE-PEG(10%)-POE microspheres with different polymer concentrations in the organic phase. A, B, C represents 16.7, 33.3 and 50.0mg/ml of polymer concentration, respectively. Size of the bar is 10 μm.*

## 4.2 Erosion and Protein Release Mechanisms of POE-PEG-POE Microspheres

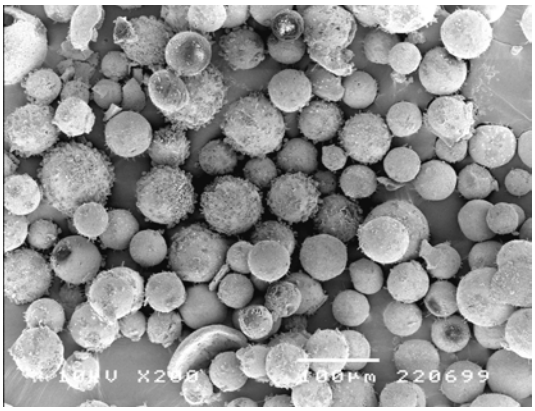
In this part, I focus on the second objective of my research work, which is to investigate polymer erosion and protein release mechanisms of POE and POE-PEG-POE microspheres. Essentially, the microspheres surface and internal morphologies during the *in vitro* degradation, the polymer degradation rate, as well as the protein release profiles were thoroughly studied.

### 4.2.1 Morphologies of Degrading Microspheres

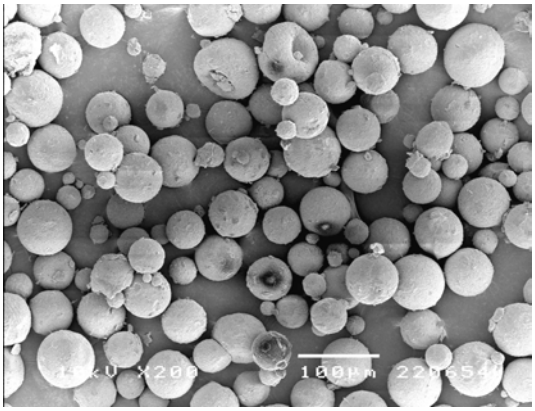
It was frequently reported that polyesters underwent bulk erosion mechanism and the microparticles degradation occurred in a heterogeneous manner [112]. In this case, the polyester breakdown products accumulated within the microparticles, leading to more rapid degradation in the center. Hence, such microparticles appeared less spherical with increased polymer degradation [7, 112]. Figure 4.18 illustrates surface SEM micrographs of POE-PEG-POE microspheres after incubation in PBS buffer at pH 7.4 for 14 weeks. Unlike polyester microparticles, POE-PEG-POE microparticles became smaller, but remained spherical even after exposure to PBS buffer for such a long period of time. The surfaces became rougher with the progression of polymer degradation (Figures 4.19, 4.20 and 4.21). The internal morphology of POE-PEG (20%)-POE microspheres shows no apparent changes before and after *in vitro* tests



**(A)**

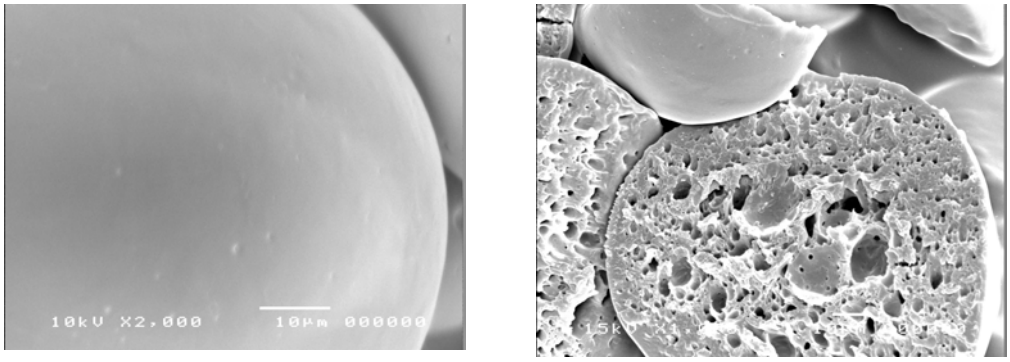


**(B)**

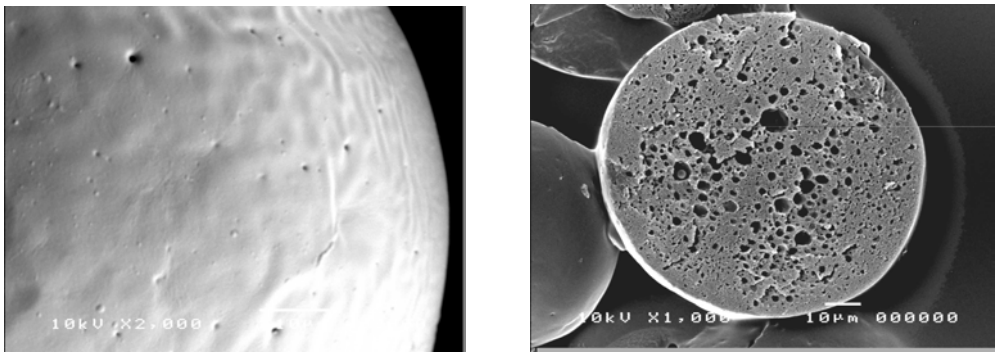


**(C)**

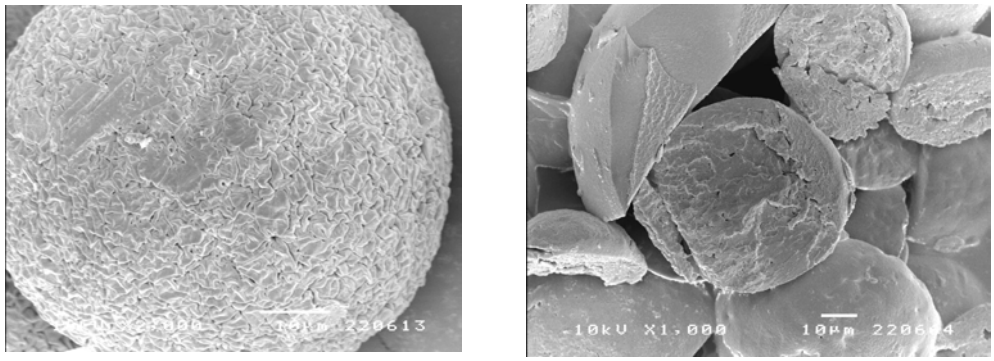
*Figure 4.18 SEM scans of POE-PEG-POE microspheres after incubation in PBS buffer, pH 7.4, 37°C for 14 weeks: (A) POE-PEG(5%)-POE, (B) POE-PEG(10%)-POE (C) POE-PEG(20%)-POE. Size of the bar is 100 μm.*




**(A)**




**(B)**




**(C)**

*Figure 4.19 SEM scans of POE-PEG(5%)-POE microspheres before (A) and after (B) 4-week (C) 14-week in vitro release. Size of the bar is 10 µm.*

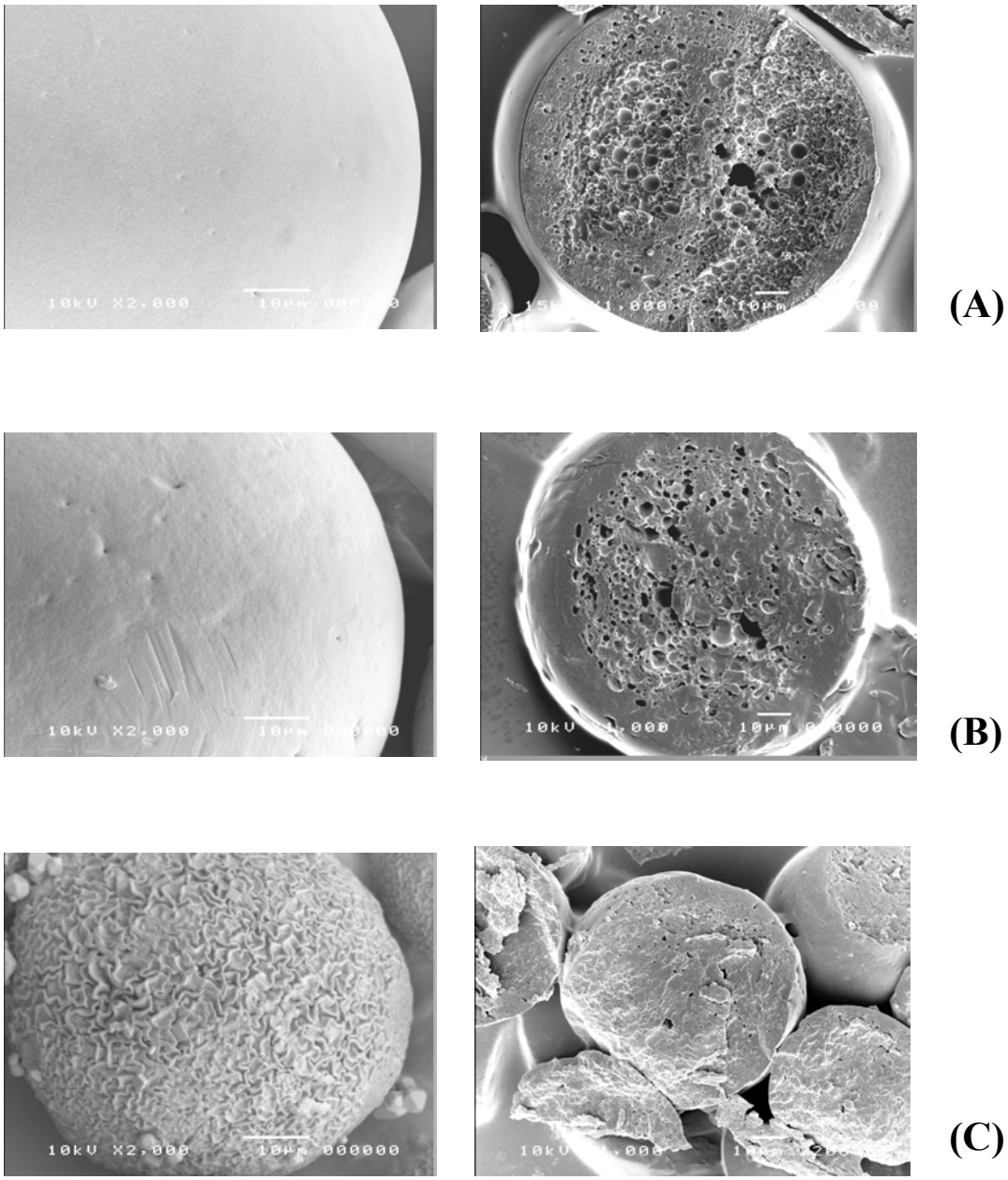
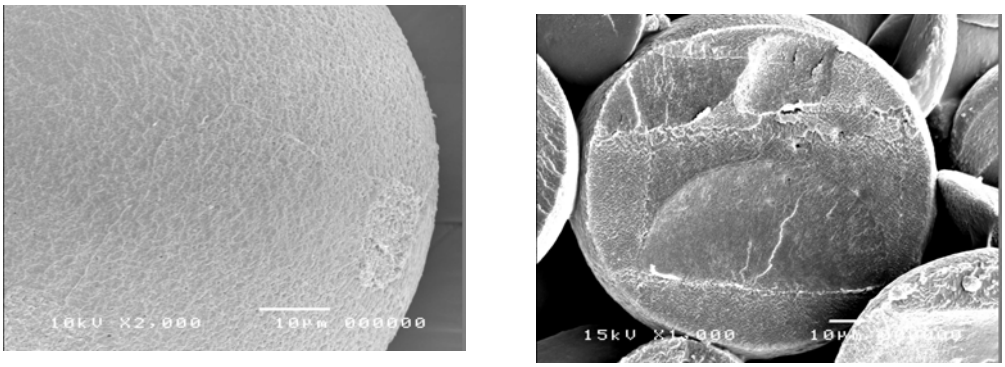
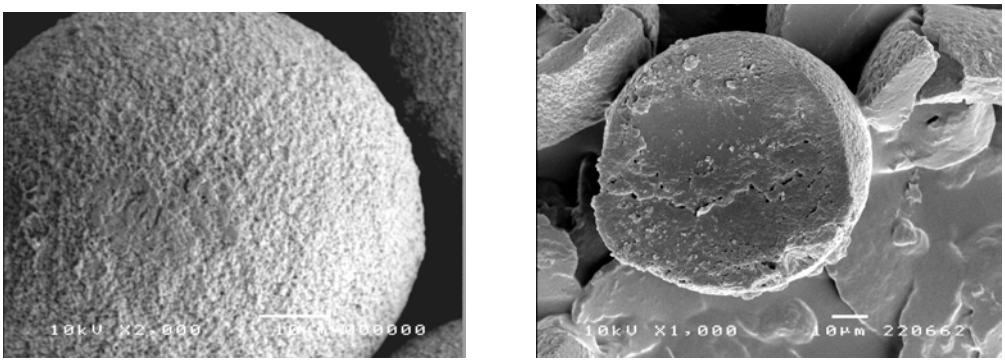


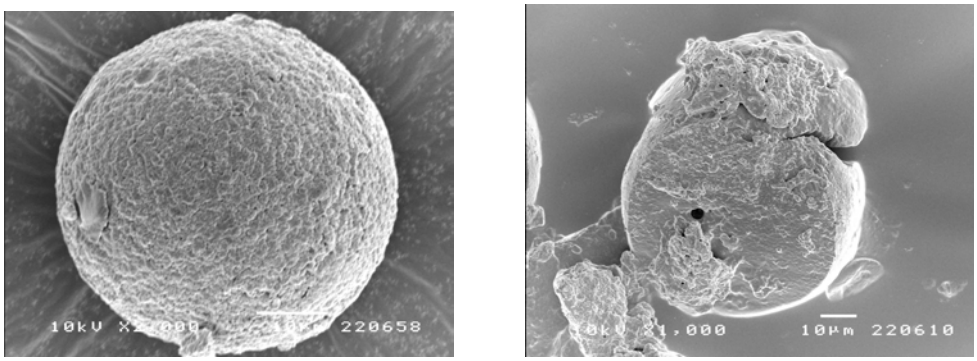

*Figure 4.20 SEM scans of POE-PEG(10%)-POE microspheres before (A) and after (B) 4-week (C) 14-week in vitro release. Size of the bar is 10 µm.*




**(A)**



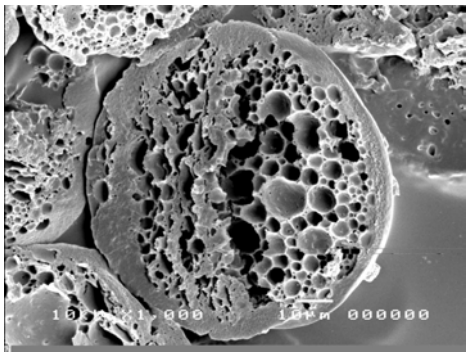

**(B)**



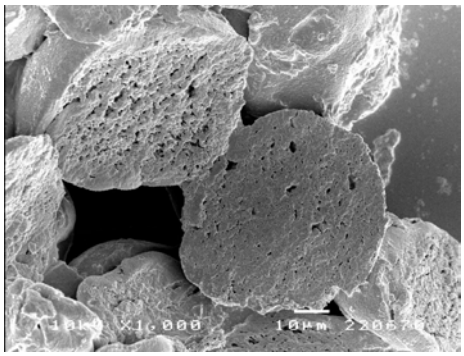

**(C)**

*Figure 4.21 SEM scans of POE-PEG(20%)-POE microspheres before (A) and after (B) 4-week (C) 14-week in vitro release. Size of the bar is 10 µm.*

**Before release**



**4-week**



**14-week**

*Figure 4.22 Internal morphology of POE-PEG(5%)-POE microspheres (DI water as the external aqueous phase) before and after in vitro release. Size of the bar is 10 μm.*

(Figure 4.21). On the other hand, POE-PEG (5%)-POE and POE-PEG (10%)-POE microspheres appeared to become denser due to the decrease in internal pore sizes (Figures 4.19 and 4.20). This possibly arises from the swell of the internal matrix because of the hydrophilic PEG segments and the loss of mechanical strength in the degrading microspheres, which might have led to the collapse of the internal porous structure during the vacuum drying process. Figure 4.22 in particular reveals that with increasing polymer degradation, the dense boundary of the microsphere surface became thinner. The findings suggest that the microspheres eroded from the surface towards the center.

### 4.2.2 Microspheres Water Uptake and Swelling

PEG is highly water-soluble. Thus, it was observed that PLGA-PEG-PLGA copolymers swelled to varying degrees depending on the PEG content [103]. Results of water uptake for POE and POE-PEG-POE microspheres are shown in Figure 4.23. Clearly, the water content within microspheres tended to increase with the increasing of PEG content. Table 4.8 demonstrates the significant swelling observed with POE-PEG (20%)-POE microspheres. The mean diameter of the microspheres increased from 90 μm to 164 μm after incubation for two days in PBS buffer. It subsequently decreased gradually and reached 93 μm on Day 14 mainly because of polymer degradation as well as the nature of densification. In contrast, POE-PEG (5%)-POE and POE-PEG (10%)-POE microspheres did not swell so much due to the relatively low PEG content. The microspheres underwent decreases in mean diameter of 11 and 14 μm, respectively, during the first two weeks. Thus, the swelling properties of the

microspheres made from each POE-PEG-POE polymer appeared to affect its rate of degradation and protein release mechanism as discussed below.

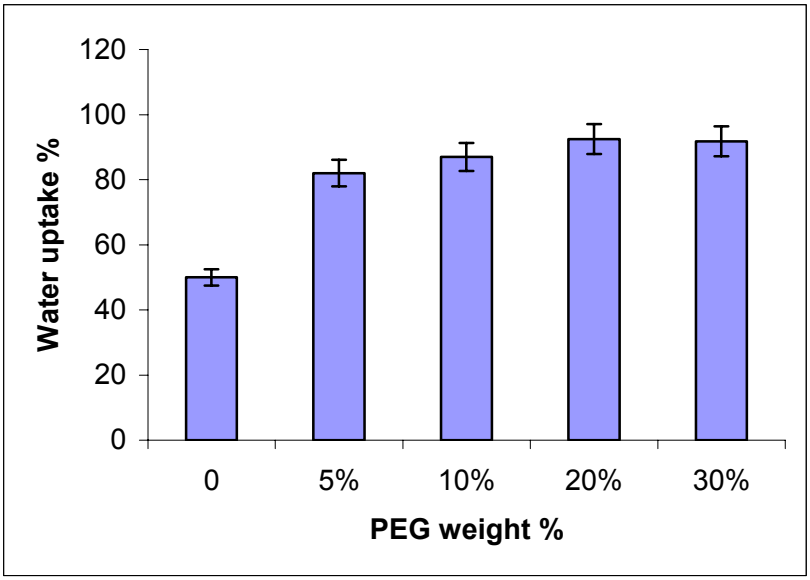



*Figure 4.23 Water uptake of POE and POE-PEG-POE microspheres after 7-day incubation in PBS at 37℃.*

*Table 4.8 Volume mean diameters (μm) of degrading microspheres*

| Samples | Before release | Day 2 | Day 4 | Day 7 | Day 11 | Day 14 |
|---|---|---|---|---|---|---|
| POE-PEG(5%)-POE | 94 | 88 | 77 | 86 | 75 | 85 |
| POE-PEG(10%)-POE | 98 | 94 | 90 | -- | -- | 84 |
| POE-PEG(20%)-POE | 90 | 164 | 133 | 142 | 100 | 93 |

### 4.2.3 pH Changes as A Function of Incubation Time

The acidity change of the release media during the *in vitro* process was monitored to investigate polymer degradation. Figure 4.24 shows that there was a higher pH drop especially for POE-PEG (5%)-POE and POE-PEG (10%)-POE during the first week

*in vitro*. Thereafter, the pH change was too little to be detected. POE employed in this study contains latent acids. After hydrolysis, acidic products were created. The higher pH drop indicated faster degradation at the initial stage. From Figure 4.24, it can also be seen that the degradation of POE-PEG (5%)-POE and POE-PEG (10%)-POE was faster than that of POE-PEG (20%)-POE during the initial stage of *in vitro* tests. This is consistent with the trend of molecular weight change and weight loss for the various polymers, which is to be reported below.

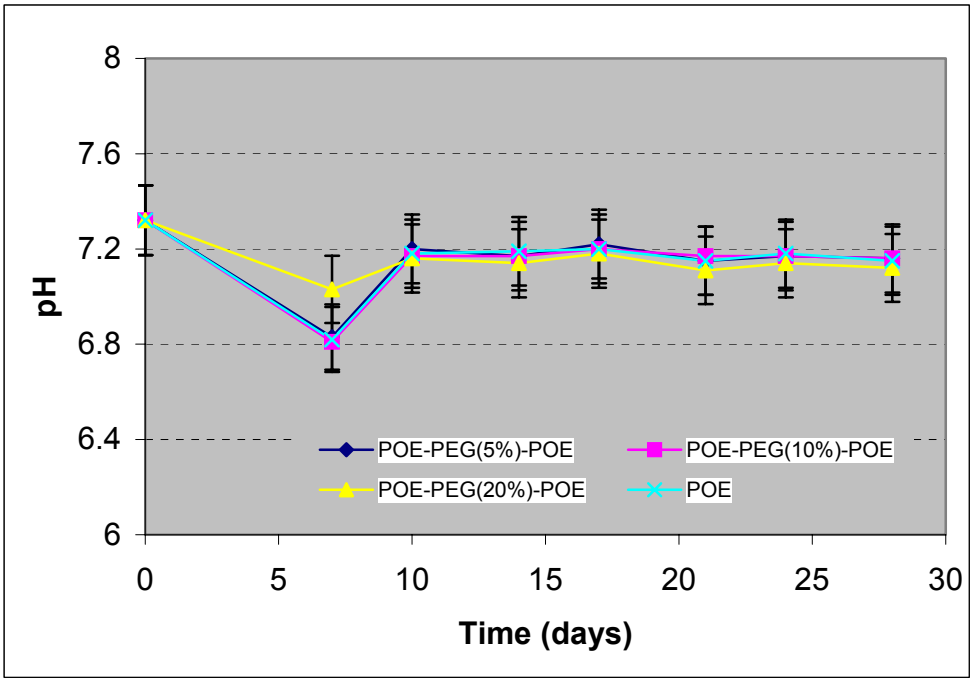



*Figure 4.24 pH changes as a function of incubation time.*

### 4.2.4 Molecular Weight Changes And Weight Loss

The changes in weight, and molecular weight of the microspheres were monitored as a function of incubation time. From Figure 4.25, it can be seen that the POE-PEG (5%)-POE and POE-PEG(10%)-POE microspheres displayed a biphasic degradation pattern, which was characterized by a high initial decrease during the first two weeks,

with subsequently much slower molecular weight loss for 12 weeks. However, the POE-PEG (20%)-POE microspheres possessed a rather constant molecular weight during degradation (Figure 4.25). For instance, during the first two weeks, weight molecular weight of the POE-PEG (5%)-POE, POE-PEG (10%)-POE and POE-PEG (20%)-POE microspheres lost 38%, 44% and 27%, respectively. From Week 2 to Week 14, an almost identical weight molecular weight loss ranging from 35% to 38% occurred in all three kinds of microspheres. Not surprisingly, changes in polydispersity index for all the triblock copolymers also followed up a similar trend with an initial increase followed by a constant value (Figure 4.26).

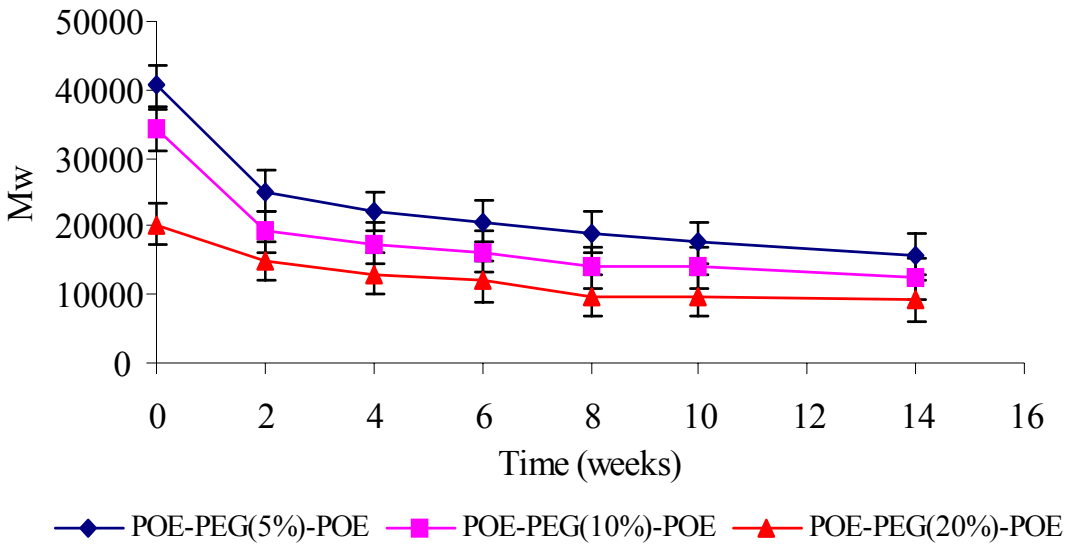



*Figure 4.25 Weight average molecular weight (Mw) changes of POE-PEG-POE microspheres as a function of incubation time.*

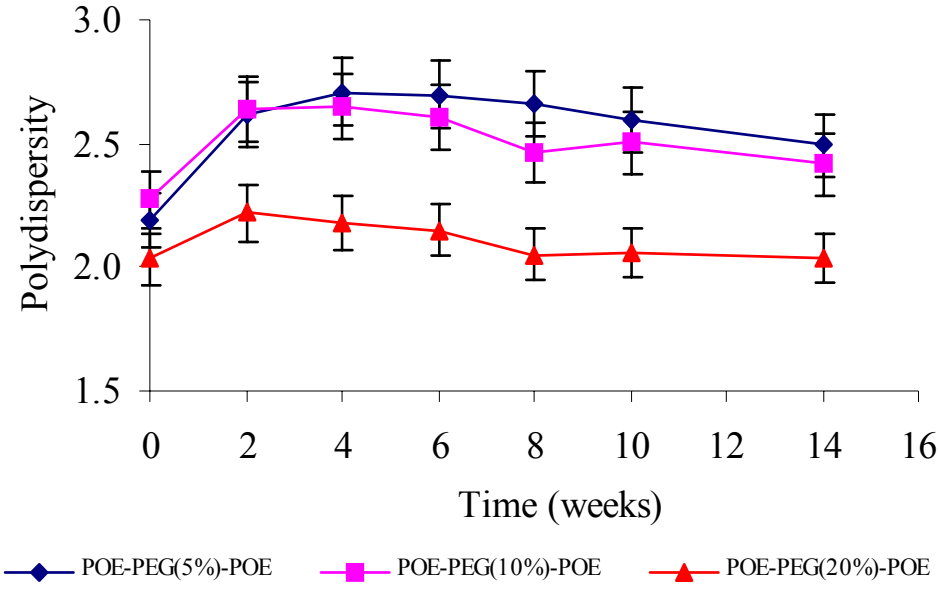



*Figure 4.26 Polydispersity index of POE-PEG-POE microspheres as a function of incubation time.*

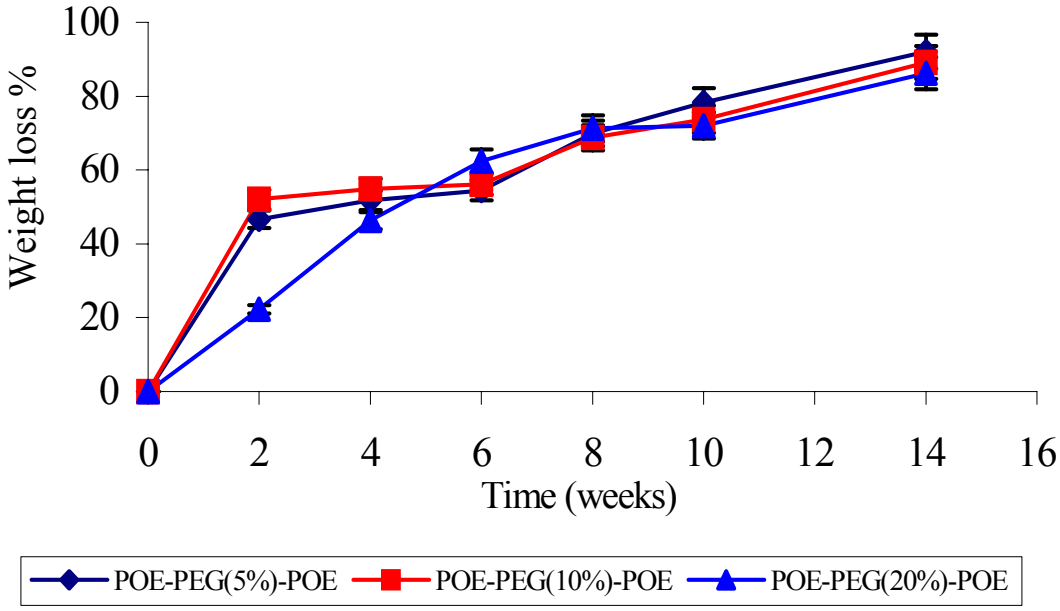



*Figure 4.27 Weight loss of POE-PEG-POE microspheres as a function of incubation time.*

According to Li *et al.* [114], there were three phases in the LPLA-PEG-LPLA polymer system when immersed in an aqueous phase: a glassy and hydrophobic LPLA phase with lowest water content, a swollen PEG phase with highest water content and a phase consisting of LPLA and PEG with a higher water content than the LPLA phase. Like LPLA-PEG-LPLA, POE-PEG-POE contains highly hydrophobic POE (CDM/TEG/TEG-diGL: 94:5:1) and hydrophilic PEG segments. When incubated in the PBS buffer, water penetrated through the microspheres matrix and preferentially stayed in PEG domains *via* hydration. Due to the microphase separation, the cleavage of POE-PEG-POE polymer occurred predominantly in the phase of POE and PEG. The triblock polymers degraded most likely between the PEG and POE blocks. Thus, preferential PEG loss from the microsphere matrix might attribute to a rapid initial degradation and weight loss.

The glass transition temperature (*Tg*) of polymeric microspheres was also monitored. *Tg* of POE-PEG (5%)-POE degrading microspheres is presented in Figure 4.28. *Tg* of both polymer and microspheres are very close (79.5 °C for polymer and 79.4 °C for microspheres), reflecting the microspheres fabrication process did not alter the length of polymer chain. *Tg* of the microspheres decreased to 74.9 and 68.0 °C after 2 and 4 weeks *in vitro*, respectively. The decrease of *Tg* resulted from the degradation of polymer and protein release. Protein release might leave some space, making the polymer chain more flexible and leading to lower *Tg*.

Interestingly, we also observe from Figure 4.25 that molecular weight decrease of the triblock copolymers with less PEG contents was faster. Possibly, the triblock

copolymers with lower PEG contents of 5% and 10% in weight yielded a less phase compatibility, which might enable the ester-bonds in the vicinity of the POE/PEG interface to break down more easily. From Figure 4.27, we observe a higher initial weight loss ranging from 47% to 52% during the first two weeks for the copolymers with low PEG contents. However, these two POE-PEG-POE copolymers only contain 5% and 10% PEG in weight, respectively. If loss of the PEG segments in the polymer backbone and the protein released mainly accounted for this initial weight reduction, the maximum loss in mass should not be more than 20%. Therefore, it enabled us to assume that a less phase compatibility of the copolymers with low PEG contents also promoted degradation of the POE blocks, resulting in a higher initial decrease in mass and molecular weight. In addition, more porous internal structure might be another reason for a faster initial degradation in POE-PEG (5%)-POE and POE-PEG(10%)-POE microspheres. From Figure 4.25, it can also be seen that the molecular weight remained rather constant with these two copolymers for 12 weeks after the initial degradation, but mass loss was continuous up to a range from 89% to 92% at Week 14. This arises most likely from POE erosion properties. The mechanism of POE microspheres erosion has been extensively studied in our laboratory. The results showed that POE microspheres eroded on surfaces including both internal and external ones that were in contact with water. There were no significant changes in molecular weight during 129-day *in vitro* tests. However, the mass loss patterns were fairly continuous and linear. In addition, during the 129-day degradation, glass transition temperatures (*Tg*) of the polymers were staying constant [115]. It is postulated that the chain length and composition of the degrading POE polymers are similar to the original non-degraded polymers. In contrast, polyesters yielded a different degradation profile in which molecular weight decrease was faster than mass

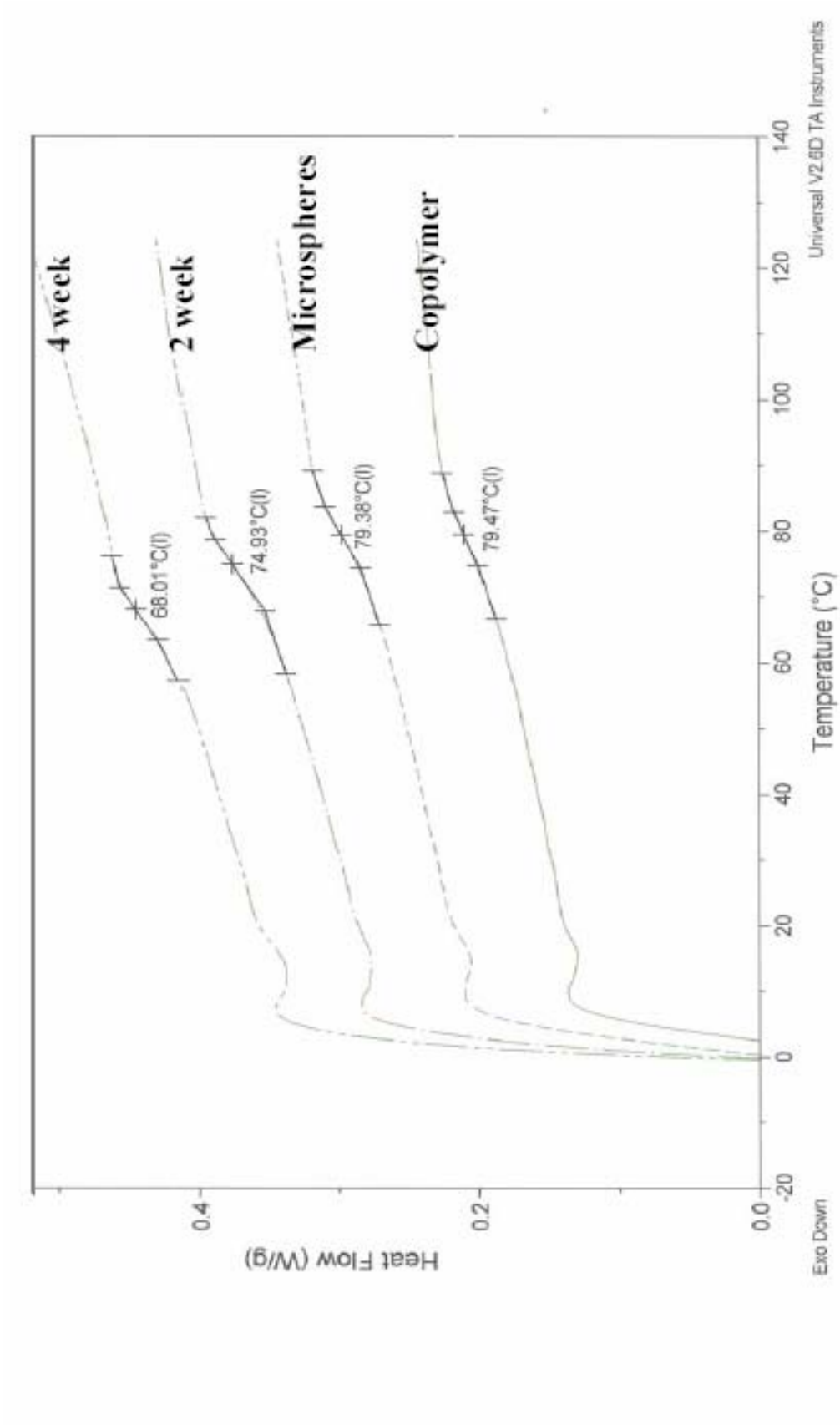



*Figure 4.28 Tg change for POE-PEG(5%)-POE microspheres.*

loss [115] since the degradation products were not small enough to dissolve or could not move out into the buffer. Thus, the degradation acids auto-catalysed polymer degradation, leading to a well-known bulk erosion mechanism.

From the experimental results presented above, at a content of 20% PEG blocks in weight, the POE-PEG-POE copolymer had a better phase compatibility and led to a steady and continuous decrease in mass during *in vitro* degradation. On the other hand, molecular weight and polydispersity index were fairly constant during the 14-week *in vitro* tests. Similarly, Li Y *et al* presented [102] that poly (L-lactide) (LPLA)-PEG- poly (L-lactide) (LPLA) yielded biphasic profiles in molecular weight decrease and weight loss. The first phase was caused by PEG loss and LPLA degradation was reflected in the second phase. However, poly (L-lactide-co-glycolide) (LPLG) and PEG blocks had a better micro-phase compatibility and thus LPLG-PEG-LPLG polymer underwent an accelerated degradation. The degradation was not dominated by PEG loss but by a loss of both PEG and LPLG blocks.

### 4.2.5 BSA Release Mechanism

Figures 4.29 and 4.30 display BSA *in vitro* release patterns from the microspheres made from POE and POE-PEG-POE polymers, respectively. It clarifies that compared to some other polymers such as polyesters [110] [113] [114], the BSA initial bursts were well controlled by POE and POE-PEG-POE polymers. POE microspheres yielded a BSA release platform over ten days (Figure 4.29). This might be due to both the polymer hydrophobicity nature and the internal structure with a thick and dense wall. Normally, it needs longer time for water to penetrate through more hydrophobic

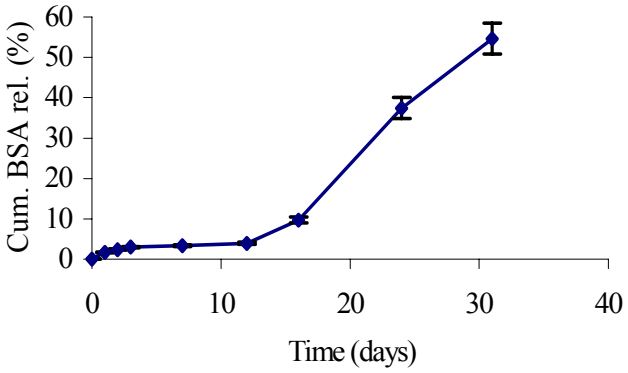



*Figure 4.29 Release profile of POE microspheres.*

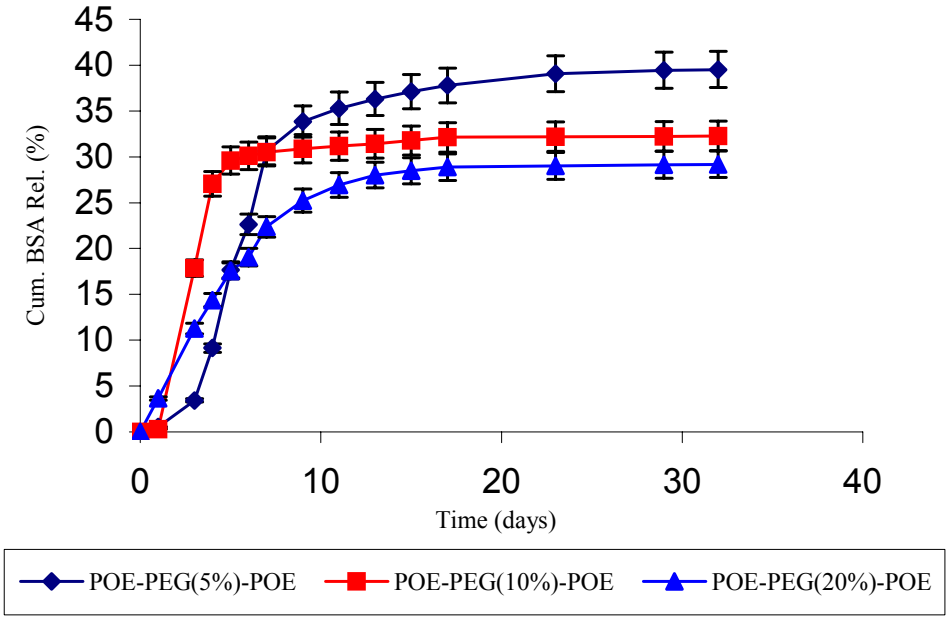



*Figure 4.30 Release profiles of POE-PEG-POE microspheres.*

polymer matrix or across the thick wall to let BSA diffuse out. From the curves in Figure 4.30, we also observe that BSA release rate increased when PEG content increased from 5% to 10%. But it decreased when PEG content continuously increased to 20%. The presence of PEG promoted water penetration, which should result in a rapid BSA release. However, a slower BSA release was obtained from the POE-PEG (20%)-POE microspheres with a higher PEG content. This is probably due to their dense structure.

As presented in previous paragraphs, the POE-PEG (5%)-POE and POE-PEG(10%)-POE microspheres lost 38% and 44% in mass, respectively, during the first two weeks. In addition, both the POE-PEG (5%)-POE and POE-PEG (10%)-POE microspheres did not show significant swelling. It can be seen from Figure 4.30 that 29 and 40% BSA are released out from the POE-PEG (5%)-POE and POE-PEG (10%)-POE microspheres, respectively, during the initial stage. Here, effect of BSA loading, porosity and size of microspheres was investigated to understand the BSA release mechanism. Figure 4.31 indicates BSA release patterns from the POE-PEG (5%)-POE microspheres fabricated at different loadings. With an increase in the BSA loading, a high BSA release rate was achieved resulting from a high driving force for BSA diffusion. A similar result was observed for POE-PEG (5%)-POE microspheres with walls (Figure 4.32). Figure 4.33 compares BSA release of the microspheres with the wall with those without wall. It was found that the presence of wall did prevent BSA release. As reported in the previous paper [115], POE-PEG (10%)-POE microspheres with various sizes and internal morphologies were produced at different polymer concentrations. Lower polymer concentrations yielded more porous and smaller microspheres. This enabled BSA to diffuse out faster (Figure 4.34). For

instance, during the first two weeks, 50%, 37% and 28% BSA was released out from the microspheres prepared at 16.7%, 33.3% and 50.0% polymer concentrations, respectively. Thus, we suggest that BSA release mechanism from the microspheres made from the POE-PEG (5%)-POE or POE-PEG (10%)-POE copolymers was based on both diffusion and erosion.

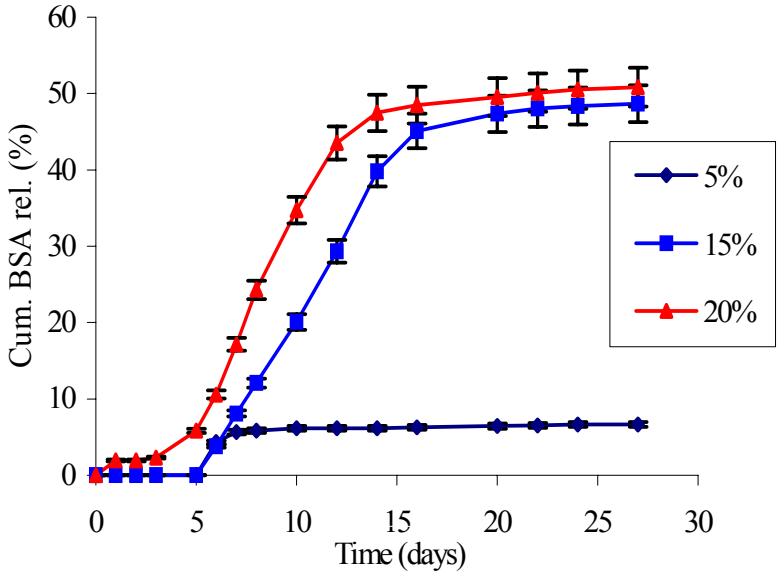



*Figure 4.31 Release profiles of POE-PEG (5%)-POE microspheres with various BSA loadings.*

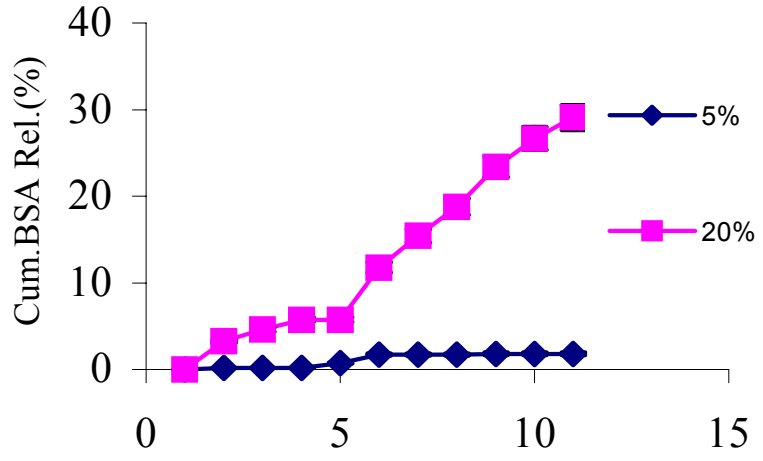



*Figure 4.32 Release profiles of POE-PEG (5%)-POE microspheres with various BSA loadings.(DI water as the external aqueous phase)*

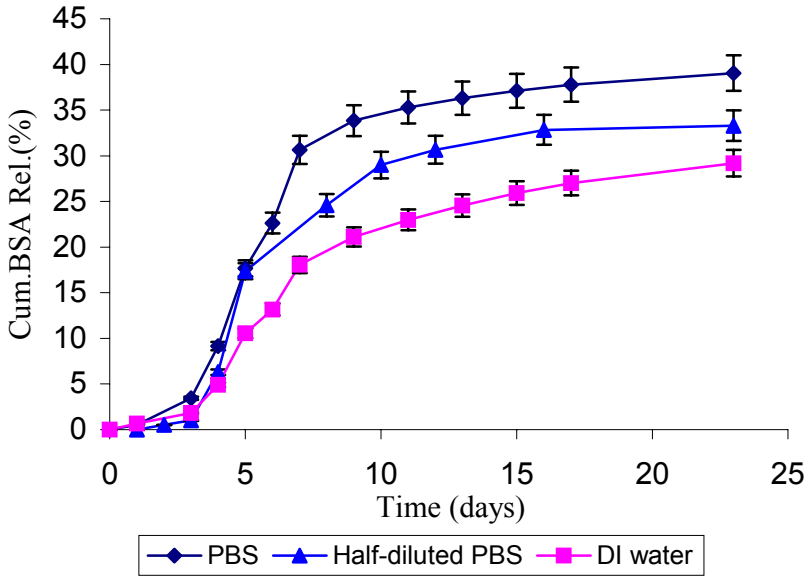



*Figure 4.33 Release profiles of POE-PEG (5%)-POE microspheres. (Using PBS, half-diluted PBS and DI water as the external aqueous phase.)*

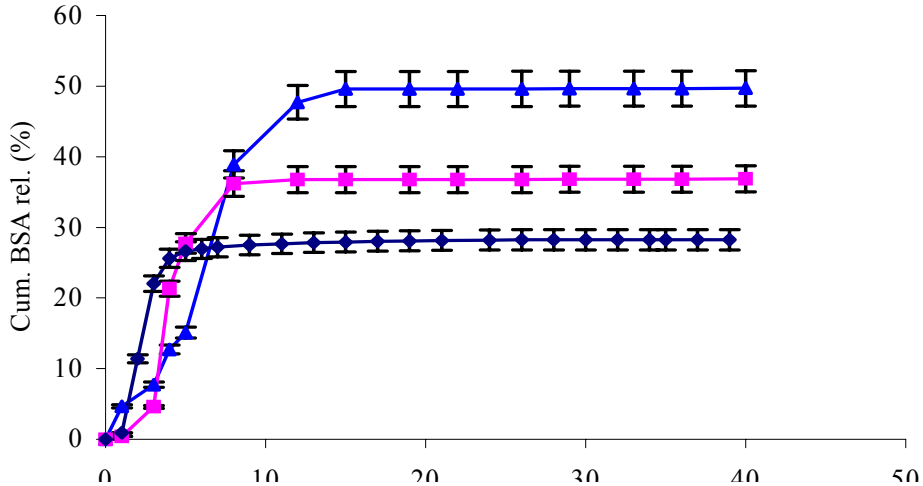



*Figure 4.34 Release profiles of POE-PEG (10%)-POE microspheres with various polymer concentrations.*

The triblock copolymer with 20% PEG in weight swelled drastically after incubated in the PBS buffer. During the first two weeks, 29% BSA release was accompanied with 22% polymer mass loss (Figures 4.27 and 4.30). Figure 4.35 displays the effect of BSA loading level on release profiles from POE-PEG(20%)-POE microspheres. It shows linear BSA release profiles. BSA release rate increased with an increase in loading level. Nearly 70% BSA was released during the first two weeks with a 20% loading. Obviously, swelling, erosion and diffusion contributed to BSA release from the POE-PEG (20%)-POE microspheres during the initial phase.

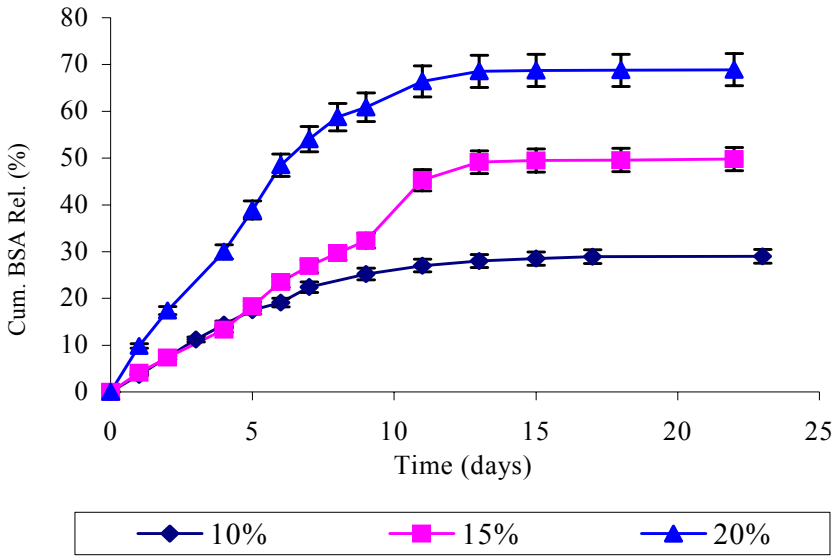



*Figure 4.35 Release profiles of POE-PEG(20%)-POE microspheres with various BSA loadings.*

From Figure 4.30, we also observe a non-release and incomplete release of BSA encapsulated within the POE-PEG-POE microspheres. During the first initial phase, 29-40% intact BSA release was confirmed with both HPLC and BCA analysis. No further BSA was detected in the *in vitro* medium up to Week 14 using both two methods although the microspheres underwent 86% to 92% weight loss. The protein non-release phenomenon was often found for PLGA polymers and PLA (PLGA)-

PEG-PLA (PLGA) copolymers containing proteins [84, 101, 117, 118]. This may be due to either protein aggregation or non-specific adsorption onto the surfaces of degrading microspheres [119]. Morlock M *et al.* [118] demonstrated an erythropoietin non-release phase with the microspheres made from poly(lactide-co-glycolide) (PLGA)-PEG-poly(lactide-co-glycolide) (PLGA) triblock copolymers containing 7-32% PEG after a 15-20% first-day burst. However, the copolymer with 46% PEG showed a 17% first-day burst followed by a continuous release to 13% erythropoietin up to Day 22. Crotts G *et al.* [86] investigated BSA stability using SDS-PAGE analysis and suggested that BSA was covalently aggregated within PLGA microspheres during polymer degradation and non-specific BSA adsorption onto polymer surface was a critical parameter to influence protein release rates.

In our research, confocal microscope was utilized to detect the BSA presence within microspheres after release (Figure 4.36). The images illustrate BSA was still present within POE-PEG (5%)-POE microspheres after 14 weeks *in vitro*. In addition, BSA was also detected with POE-PEG (10% and 20%)-POE microspheres using FTIR (Figure 4.37). The carbonyl band from the polymer, amide carbonyl as well as carboanion bands from BSA did not shift significantly. No new bands appeared during the degradation process. Besides, BSA was detected in the microspheres even after 14-week *in vitro* degradation. Similar results were also observed with the POE-PEG (5%)-POE and POE-PEG (10%)-POE microspheres. Table 4.9 lists the chemical compositions of the degrading microsphere surface. A BSA molecule contains a number of nitrogen atoms. No nitrogen atoms were found on the surface of the degrading microspheres before Week 2. However, it was detectable after 4-week degradation and the percentage of nitrogen atoms slightly increased as a function of

degradation time. With microspheres degradation, surface area enlarged, which might lead to more BSA adsorption.

*Table 4.9 Atomic percentage of elements on the surface of degrading POE-PEG (20%)-POE microspheres*

| Samples | C% | O% | N% |
|---|---|---|---|
| Before release | 72.6 | 27.4 | 0.0 |
| After 2-week | 76.1 | 23.9 | 0.0 |
| After 4-week | 74.4 | 23.3 | 2.3 |
| After 6-week | 74.5 | 23.0 | 2.5 |
| After 8-week | 74.0 | 21.9 | 4.2 |
| After 10-week | 73.0 | 21.4 | 5.6 |

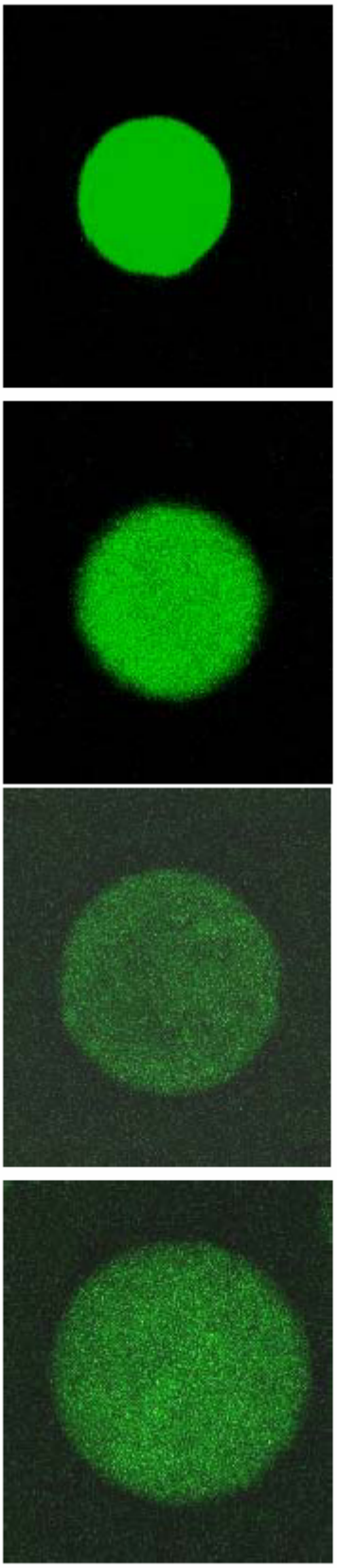



*Figure 4.36 Protein distribution within POE-PEG(5%)-POE microspheres before and after release.*

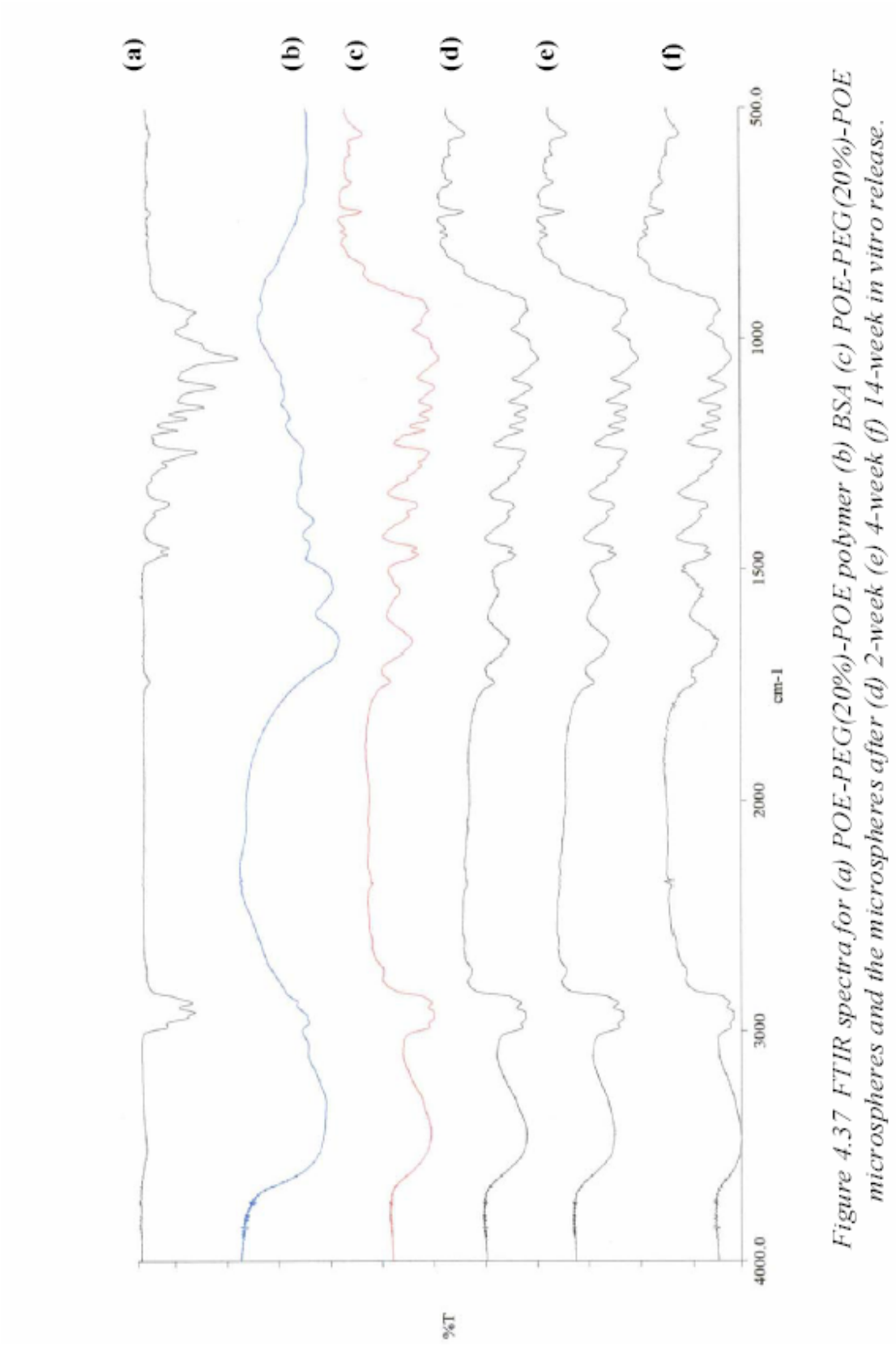



*Figure 4.37 FTIR spectra for (a) POE-PEG(20%)-POE polymer (b) BSA (c) POE-PEG(20%)-POE microspheres and the microspheres after (d) 2-week (e) 4-week (f) 14-week in vitro release.*

Non-reducing SDS-PAGE of BSA within POE-PEG-POE microspheres was performed to investigate BSA structural integrity. Under non-reduced conditions, all aggregates linked by disulfide bonds should remain. Figure 4.38 displays non-reducing SDS-PAGE results of standard BSA and BSA within the degrading microspheres. Faint BSA bands in response to the microspheres before release was observed due to presence of a dense skin layer that hindered BSA migration from the microspheres to the gel. From Figure 4.38, it can be seen that stained bands representing molecular weight of BSA monomer, dimmer and higher order aggregates existed in both the BSA standard and BSA within the degrading microspheres. It was reported that the double emulsion process could cause the formation of protein aggregates [86, 120]. There was no degradation during the first 8 weeks of incubation but BSA degradation bands appeared after 10 weeks. This suggests that the POE-PEG-POE copolymeric microspheres provided BSA a friendly environment in the *in vitro* medium at 37 °C at least for 8 weeks. POE blocks used in this study contain 94% rigid *trans*-cyclohexanedimethanol (CDM) and the microspheres degraded slowly after most PEG blocks left from the chains of POE-PEG (5%)-POE and POE-PEG (10%)-POE polymers. Thus, it is assumed that prior to degradation, non-release was attributed to slow polymer degradation. As for POE-PEG (20%)-POE microspheres, low degradation rate came from their dense structure. Slow release led to low BSA concentrations in the *in vitro* medium, which were not detectable by both HPSEC and BCA methods.

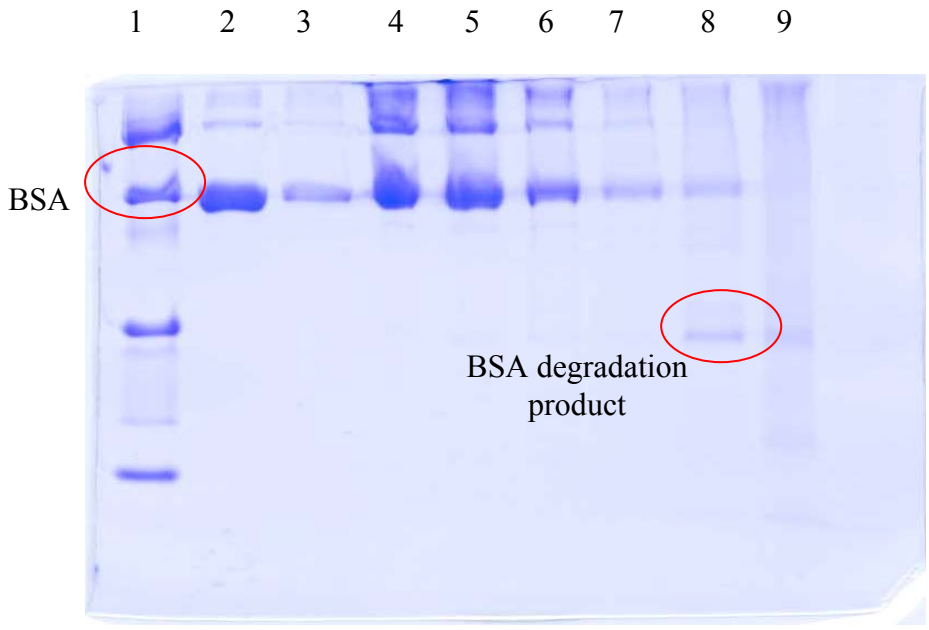



*Figure 4.38 Non-reducing SDS-PAGE results of BSA within POE-PEG(5%)-POE microspheres suspended in gel loading buffer. Lane 1, protein molecular weight markers; lane 2, BSA control; lane 3, the microspheres before release; lane 4-9, the microspheres after 2, 4, 6, 8, 10 and 14 weeks release.*

## 4.3 Modulation of Protein Release

### 4.3.1 Background

In many cases, designing a system with zero-order drug release kinetics is most desired. To meet this objective, numerous design variations have been proposed. For instance, Lee and Robinson *et al.* [121] have suggested several parameters for the drug release design. These parameters include the disease property, drug physicochemical and biological properties as well as design of drug delivery systems. The selection of polymers is critical to designing drug delivery systems.

In our previous research, we have demonstrated that protein release from POE-PEG(Mn 4,600)-POE microspheres was affected by many factors including polymer degradation rate, which largely depended on physicochemical properties of polymer such as molecular weight, hydrophilicity and compositions [122]. Processing conditions employed during the preparation of microspheres also influence protein release, which include particle size and morphology, protein encapsulation efficiency, as well as drug distribution [4]. In our research, experimental parameter changes could be an attractive approach to modify the microspheres properties as well as the release profiles of protein from microspheres [123, 124].

In this part, I focus on modulation of protein release by changing the molecular weight of PEG and POE composition. In the following paragraphs, BSA-loaded microspheres were fabricated using POE-PEG-POE with PEG having an average number molecular weight of 1,000 as well as POE-PEG-POE with a POE

composition of 1,2-PrD/1,2-PrD-diGL and CDM/TEG-diGL. Protein release from the microspheres was investigated.

### 4.3.2 Effect of PEG Molecular Weight

Although there are lots of studies on ABA (B: PEG) microspheres, few people have looked into the effect of PEG molecular weight. In my project, new POE-PEG-POE polymers with PEG having Mn of 1,000 were synthesized and utilized to fabricate BSA-loaded microspheres. The PEG content was 5%, 10% and 20% in weight.

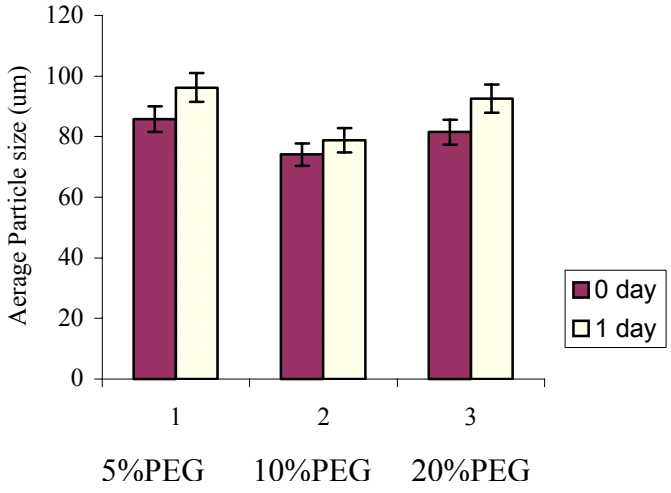



*Figure 4.39 Average mean diameter of POE-PEG (Mn 1,000)-POE microspheres before and after one-day in vitro.*

Figure 4.39 illustrates the average size of microspheres before and after the first day *in vitro* release. Due to the hydrophilicity of PEG, all the batches of POE-PEG (Mn 1,000)-POE microspheres swelled after immersed in the buffer.

Weight loss and molecular weight change of the POE-PEG (Mn 1,000)-POE microspheres were monitored paralleling to the *in vitro* study. The results are showed in Figures 4.40 and 4.41. The trends of weight loss and molecular weight change in

POE-PEG(Mn 1,000)-POE microspheres were similar to those in POE-PEG (Mn 4,600)-POE microspheres reported in *Section 4.2*. The POE-PEG (Mn 1,000)-POE microspheres with 5% and 10% PEG experienced a biphasic degradation pattern, characterized by a fast initial weight loss and a rapid decrease in molecular weight followed by a slow and constant loss in weight and molecular weight. However, the weight loss of POE-PEG (Mn 1,000)-POE microspheres with 20% PEG was more sustained, and its molecular weight was relatively constant. Changes in polydispersity index also followed a similar trend with an initial increase followed by a constant value (Figure 4.42)

The results obtained from POE-PEG (Mn 1,000)-POE copolymers supported our previous hypothesis that POE-PEG-POE with 20% PEG possessed a better compatibility between POE and PEG blocks.

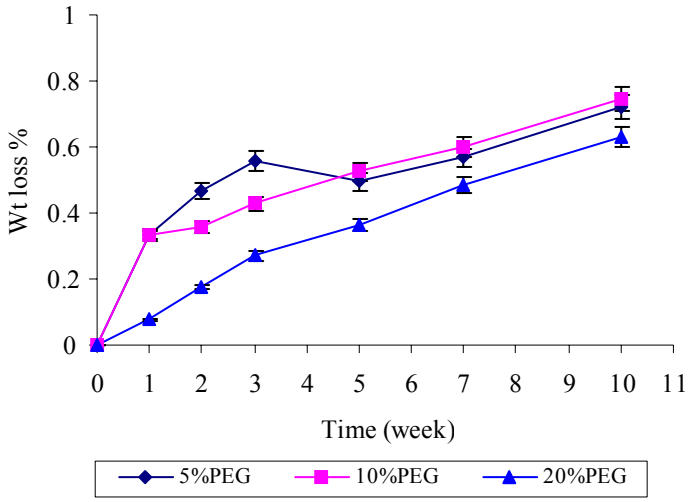



*Figure 4.40 Weight loss of POE-PEG (Mn 1,000)-POE microspheres as a function of incubation time.*

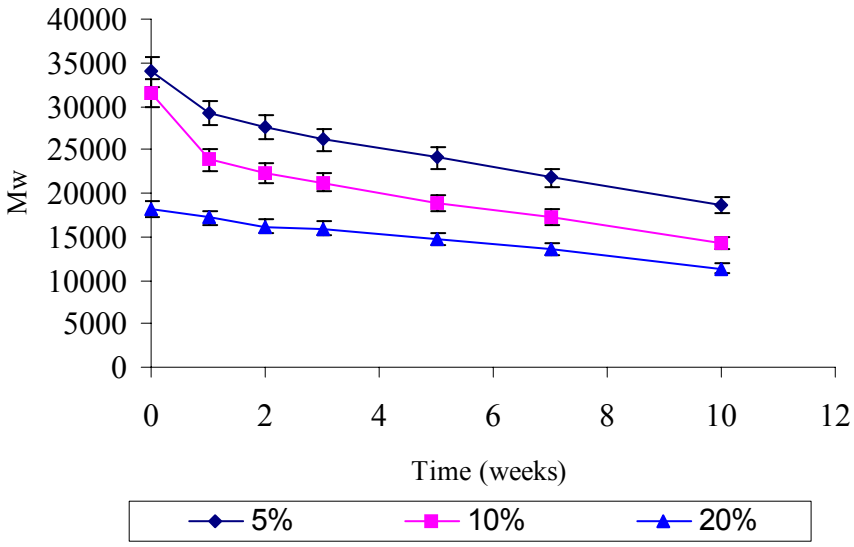



*Figure 4.41 Weight average molecular weight change of POE-PEG (Mn 1,000)-POE microspheres as a function of incubation time.*

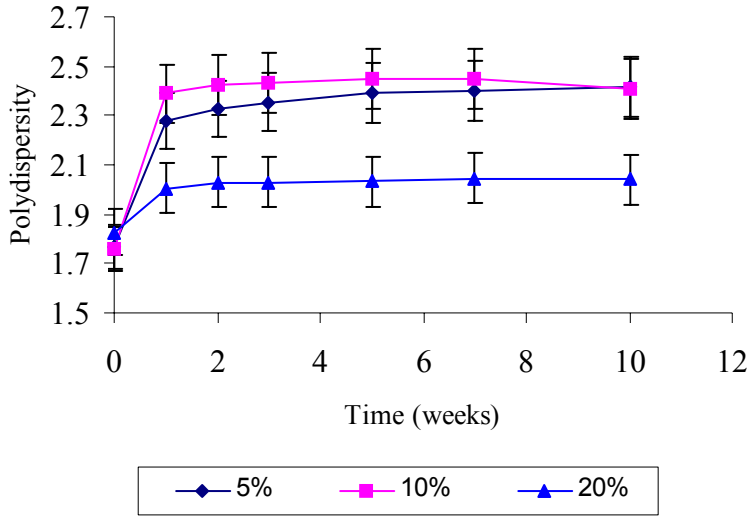



*Figure 4.42 Polydispersity index of POE-PEG (Mn 1,000)-POE microspheres with various PEG contents.*

The release profiles of POE-PEG (Mn 1,000)-POE microspheres are shown in Figure 4.43. Clearly, for POE-PEG (Mn 1,000)-POE microspheres, larger amount of BSA was released out compared to POE-PEG (Mn 4,600)-POE microspheres (Figure 4.30). Taking POE-PEG (5%)-POE as an example, the cumulative BSA release was about 27% for PEG 4,600 while it increased to about 45% for PEG 1,000 microspheres. The possible reason is that POE-PEG (Mn 1,000)-POE microspheres had looser internal structure than the POE-PEG (Mn 4,600)-POE ones (Figure 4.44).

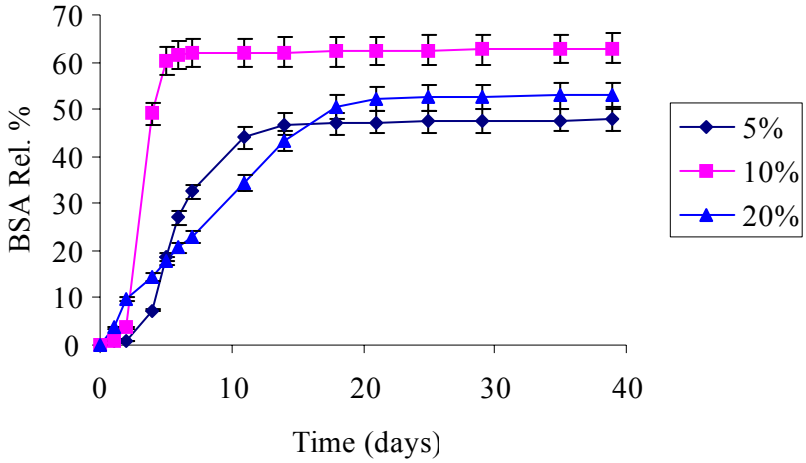
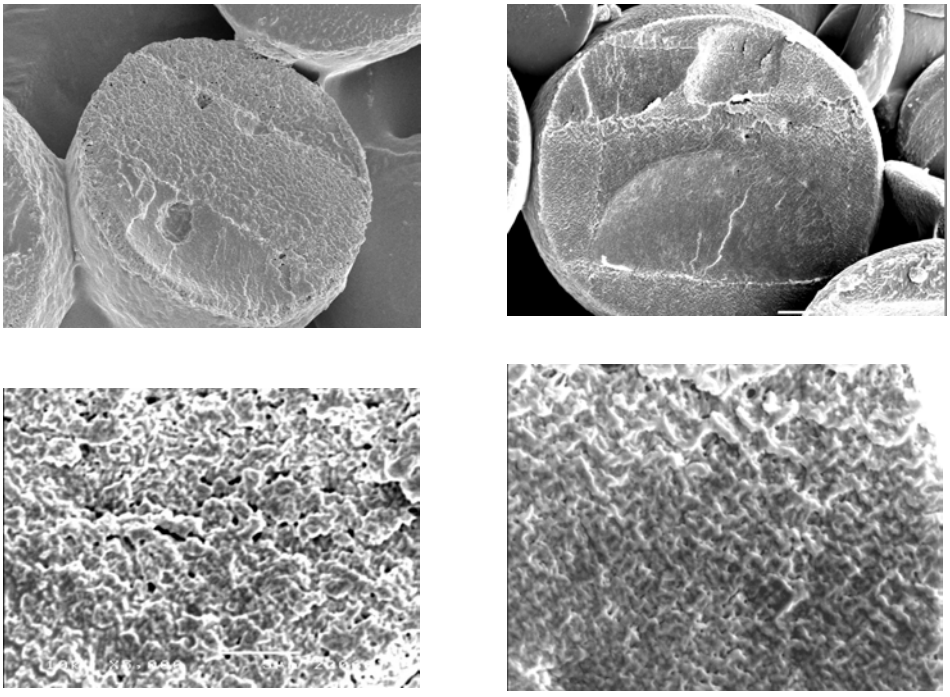



*Figure 4.43 Release profiles of POE-PEG (Mn 1,000)-POE microspheres in PBS, pH 7.4, 37°C. BSA loading is 10%.*

From Figure 4.43, it can also be observed that the microspheres made from POE-PEG (Mn 1,000)-POE with a PEG content of 20% provided a more sustained and linear BSA release profile compared to the copolymers with PEG contents of 5% and 10%. The linear and constant BSA release lasted 21 days, which is much longer than what POE-PEG (Mn 4,600)-POE microspheres provided. The cumulative BSA release could be enhanced when using 0.5 ml PVA/PBS (instead of 0.3 ml) as the internal water phase (Figure 4.45).



A B

*Figure 4.44 Internal structure of (A) POE-PEG (Mn 1,000)-POE and (B) POE-PEG (Mn 4,600)-POE microspheres. Sizes of the bars are10 μm and 5 μm, respectively.*

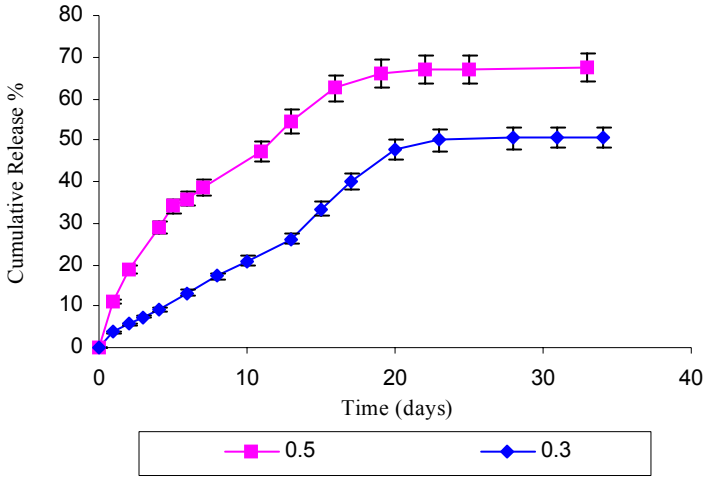



*Figure 4.45 BSA release from POE-PEG (Mn 1,000)-POE microspheres with different internal water phases.*

### 4.3.3 Effect of POE Compositions

To achieve complete protein release from ABA (B: PEG) triblock copolymers, an alternative approach is to employing block A with less rigid structure or greater hydrophilicity such that water penetration could be faster. T. Kissel *et al.* reported that when the content of glycolic acid in PLGA blocks increased, the PLGA-PEG-PLGA microspheres yielded faster and more complete EPO and FITC-dextran release [7, 119, 125].

#### 4.3.3.1 POE Composition: 1,2-PrD/1,2-PrD-diGL

In this study, POE with a composition of 1,2-PrD/1,2-PrD-diGL was employed. PEG has an average number molecular weight of 1,000. Compared to CDM, 1,2-PrD is more flexible and hydrophilic. It was expected that water penetration rate through the polymer matrix would be higher. The properties of new POE-PEG-POE copolymers are listed in Table 4.10.

The surface and internal morphology of resultant microspheres prepared from the new POE-PEG-POE copolymers were observed using SEM. The micrographs are shown in Figures 4.46 and 4.47. The porous surface was observed in the POE-PEG (1,000, 5%)-POE microspheres (A, B and C). For POE-PEG (1,000, 20%)-POE microspheres (D, E and F) with the same composition, the surface was smoother. The porous surface for A, B and C microspheres might result from their lower molecular weight and inherent viscosity. In addition, the internal structure of A, B and C microspheres were more porous, and the D, E and F micropsheres with 20% PEG were relatively

dense. This phenomenon was also observed in the previous microspheres with the POE composition of CDM/TEG/TEG-diGL (94:5:1).

*Table 4.10 Properties of POE-PEG (Mn 1,000)-POE copolymers (POE: 1,2-PrD/1,2-PrD-diGL)*

| Sample | POE Composition (1,2-PrD:1,2-PrD-diGL) | PEG % | Inherent Viscosity (dL/g) | Mw |
|---|---|---|---|---|
| A | 95:5 | 5 | 2.10 | 30553 |
| B | 90:10 | 5 | 2.32 | 29877 |
| C | 85:15 | 5 | 2.55 | 28086 |
| D | 95:5 | 20 | 2.65 | 35031 |
| E | 90:10 | 20 | 2.96 | 34138 |
| F | 85:15 | 20 | 3.17 | 34283 |

Figure 4.48 shows BSA encapsulation efficiency for all the copolymers. POE-PEG (Mn 1,000)-POE microspheres with 20% PEG yielded slightly higher encapsulation efficiency than those with 5% PEG. This is due to the more stable first emulsion (Figure 4.49) as well as stronger BSA-polymer affinity, preventing protein loss during the fabrication process.

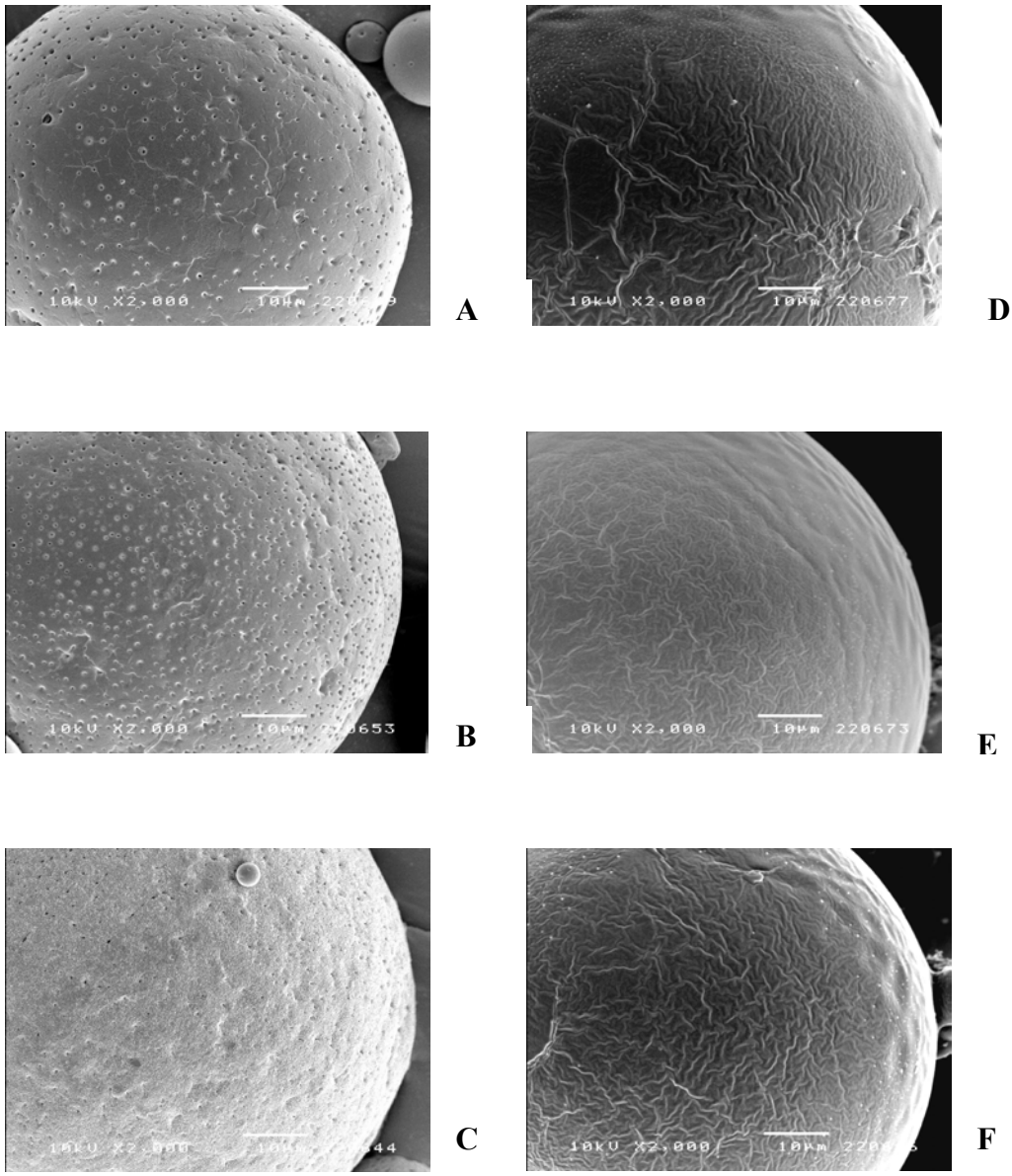



*Figure 4.46 Surface morphology of the A, B, C, D, E and F microspheres. Size of the bars is 10 μm.*

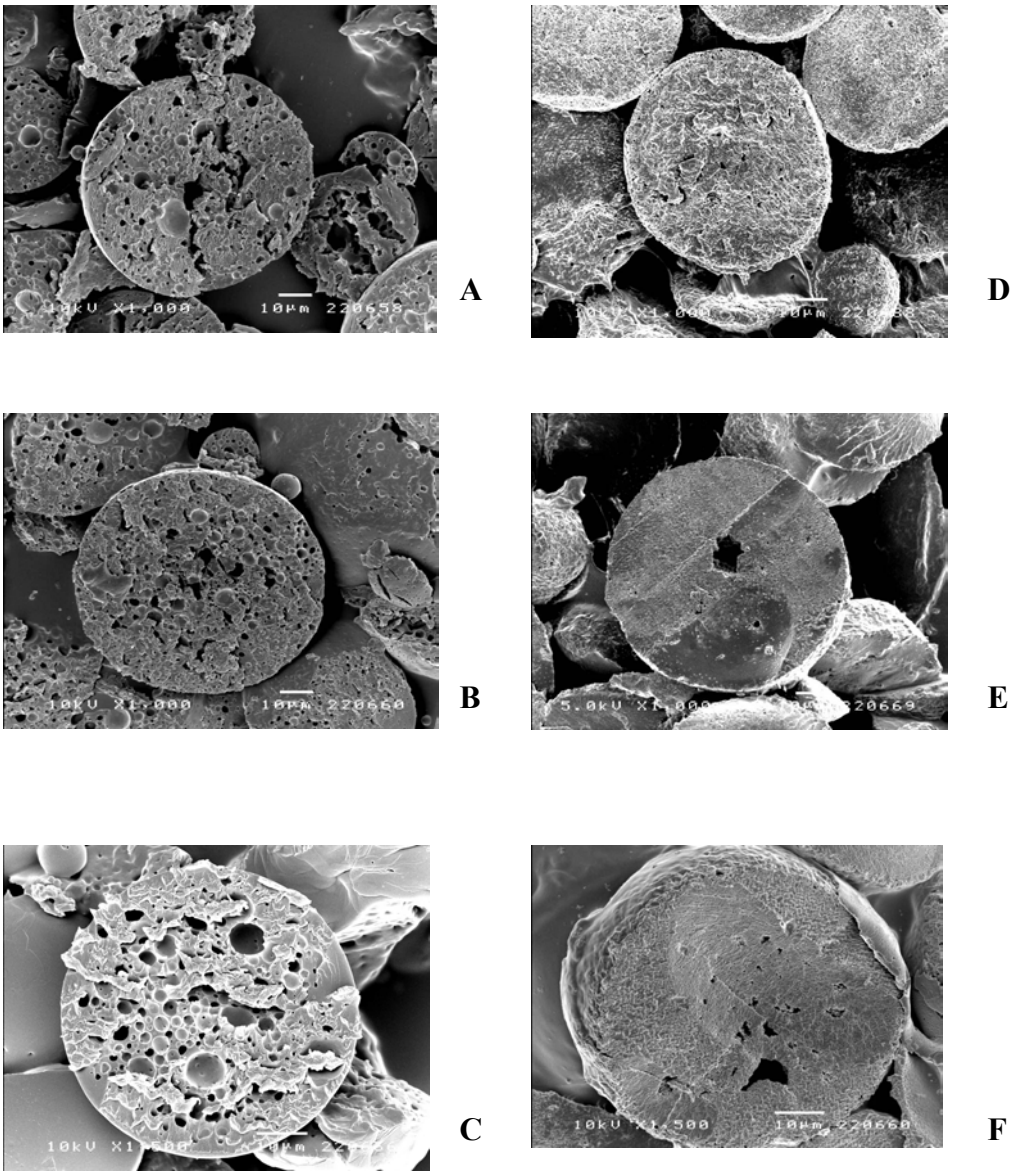



*Figure 4.47 Internal structure of A, B, C, D, E and F microspheres. Size of the bars is 10 μm.*

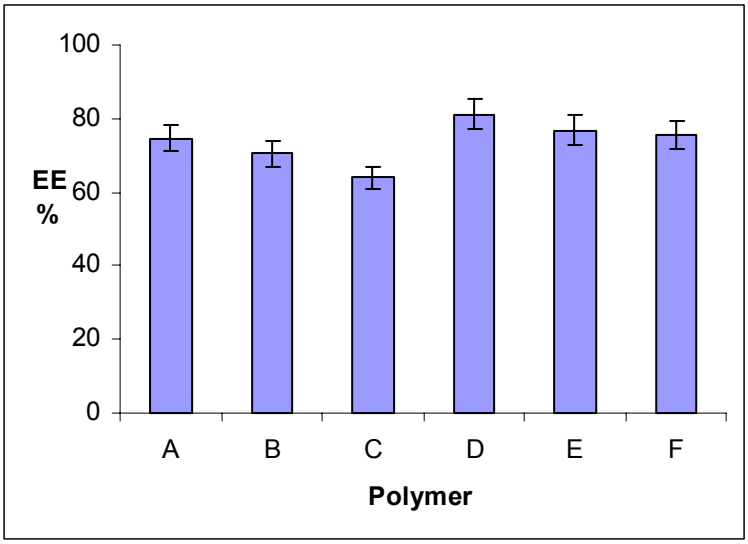



*Figure 4.48 BSA encapsulation efficiency of A, B, C, D, E and F microspheres.*

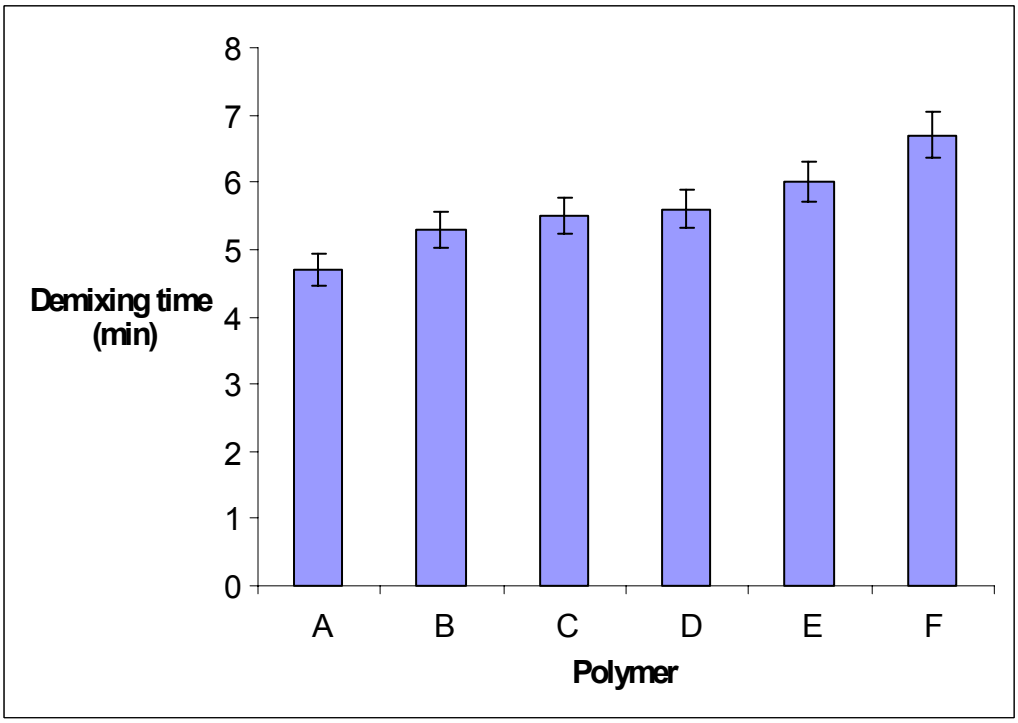



*Figure 4.49 Demixing time of the first emulsion of A to F polymers*

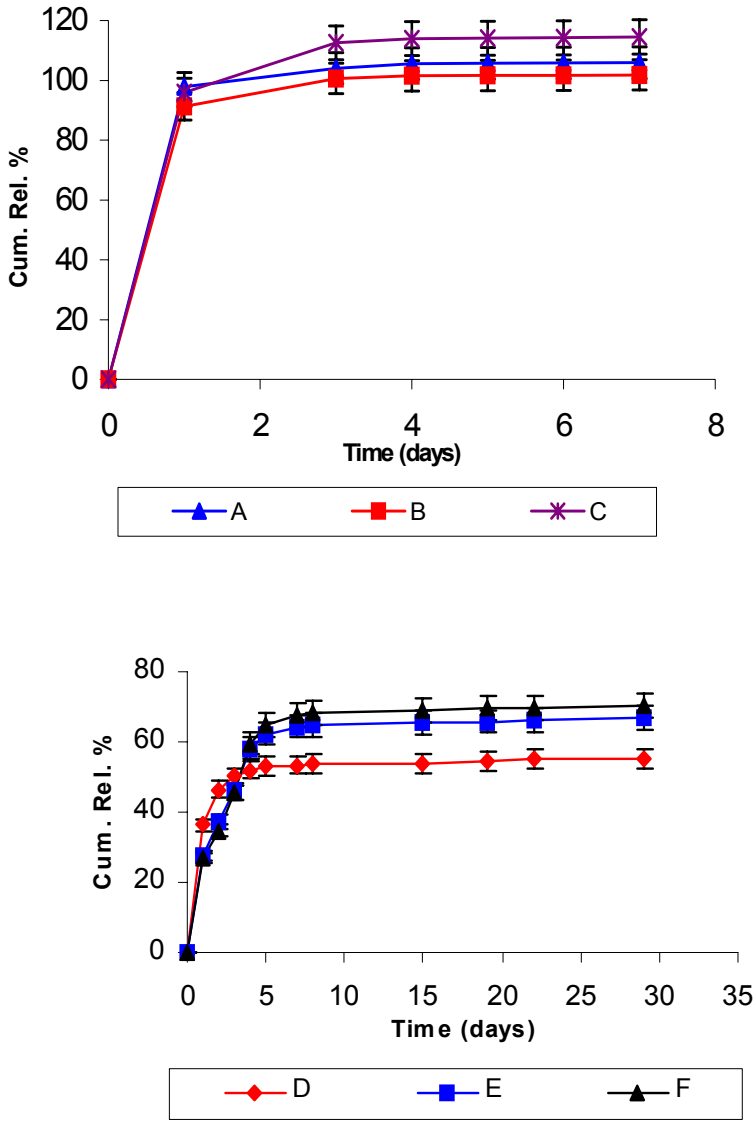



*Figure 4.50 BSA release from the POE-PEG (Mn 1,000)-POE microspheres in PBS (pH 7.4) at 37°C. BSA loading is 10%.*

BSA release profiles from the microspheres are shown in Figure 4.50. Compared to POE-PEG (Mn 1,000)-POE microspheres with a POE composition of CDM/TEG/TEG-diGL (94/5/1), POE (1,2-PrD/1,2-PrD-diGL) yielded much faster BSA release. In addition, more BSA was released out from A, B and C microspheres. This mainly resulted from their porous structure. However, BSA release from D, E and F microspheres lasted for about 5 days.

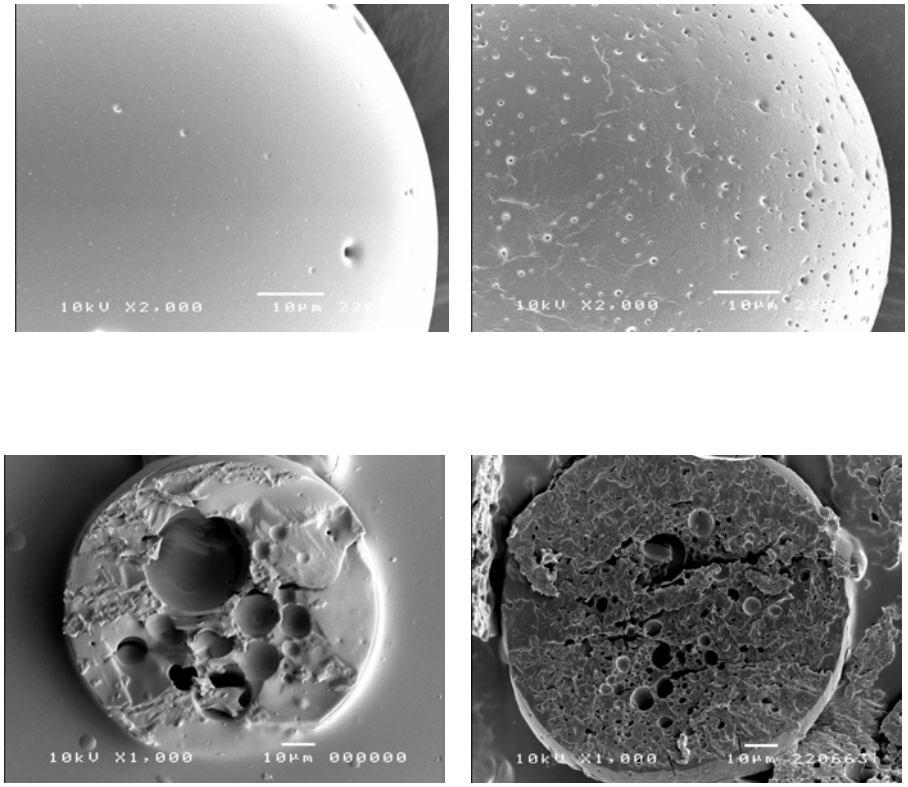
#### 4.3.3.2 POE Composition: CDM/TEG-diGL

Another two copolymers based on PEG (Mn, 1,000) were synthesized with POE (CDM/TEG-diGL) containing different amount of TEG-diGL (Table 4.11), which were employed to investigate the effect of polymer composition on protein release.

*Table 4.11 Properties of POE-PEG (5%, Mn 1,000)-POE copolymers (POE: CDM/TEG-diGL).*

| Sample | POE composition | PEG % | Inherent Viscosity (dL/g) | MW |
|---|---|---|---|---|
| G | CDM/TEG-diGL (95:5) | 5 | 2.89 | 25856 |
| H | CDM/TEG-diGL(85:15) | 5 | 3.12 | 26445 |

The CDM/TEG-diGL (85:15)-based polymer yielded much higher BSA encapsulation efficiency (80%) compared to the CDM/TEG-diGL (95:5)-based polymers (42%). It may be due to the slow solidification and unstable first emulsion. As we can see from Figure 4.51, big pores were observed in the G microspheres, resulting from the unstable first emulsion. However, the H microspheres had a uniform internal structure.




**G** **H**

*Figure 4.51 Surface and internal morphology of G and H microspheres. Size of the bars is 10µm.*

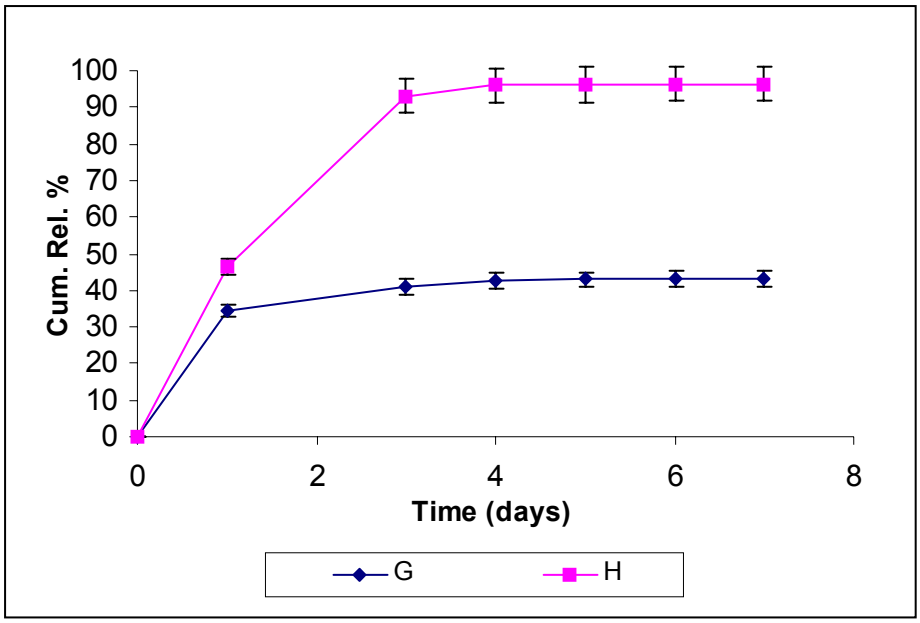



*Figure 4.52 BSA release from G and H microspheres. BSA loading is 10%.*

BSA release from the H microspheres was sustained over 3 days, and much more BSA came out during the *in vitro* course compared to the G microspheres.

Therefore, the composition of POE should be well-designed in order to achieve a desired protein release profile.

# CHAPTER FIVE

# RESULTS AND DISCUSSION 2:

# POE/PEG BLEND MICROSPHERES

The use of polymer blends as drug carriers represents an appealing approach for controlled release applications. Blending of homopolymers presents a powerful alternative, which is superior in terms of simplicity and low cost compared to copolymerization. Various blends have been reported useful for drug delivery applications [126-128]. The stability, morphology, and performance of polymer blends are intimately connected to the polymer miscibility. Miscible polymers form blends with properties analogous to those of a single-phase material. Most polymer systems are however non-compatible resulting in non-uniformity or phase separation [129]. Depending on the thermodynamic compatibility of the two chosen polymers, phase-separated polymers can be obtained, imposing different morphologies and matrix characteristics and different drug release rates. Also, the degradation and release performance can be controlled through varying component ratios. It was reported that PLGA blended with PEG yielded better release profiles of proteins compared to neat PLGA [101] [103].

In this part, I focus on POE/PEG blend microspheres for protein delivery. The microspheres are fabricated by the double emulsion method. The objective of this study is to investigate the effect of polymer blends on particle size, surface morphology, encapsulation efficiency and BSA release mechanism of the microspheres.

## 5.1 Microspheres Characterization

### 5.1.1 Particle Size Distribution

The average particle size of POE/PEG(MW 4,600), POE/PEG(MW 100,000) and POE/PEG(MW 200,000) blend microspheres immediately after fabrication is listed in Table 5.1, 5.2 and 5.3. For the POE/PEG (MW 4,600) blends, with the increasing of PEG content, particle size decreased because of the dissolution of PEG and less viscous polymer solution during the fabrication process. 10% PEG content yielded the biggest microspheres due to the highest inherent viscosity of polymer solution. For the POE/PEG (MW 100,000 and 200,000) blends, an increased PEG content also produced bigger microspheres although the polymer solution was more viscous at high PEG contents (Table 5.4a, 5.4b and 5.4c). This was probably because PEG loss in the external aqueous phase was a dominant factor. Interestingly, the diameter of blend microspheres was similar to that of the microspheres made from POE-PEG-POE block copolymers. This was because their inherent viscosities of polymer solutions were very closed (Table 5.4a, Table 4.1).

### 5.1.2 Thermal Properties

The *Tg* values of blank microspheres are listed in Table 5.5a, 5.5b and 5.5c. POE blank microspheres exhibited a *Tg* of about 104 ºC and PEG (MW 4,600) polymer showed a *Tm* of 63 ºC, PEG (MW 100,000 and 200,000) with a slightly higher *Tm* which is around 65.5 ºC resulting from increased molecular weights. For all POE/PEG blend microspheres, down shifts in both *Tg* and *Tm* by 2-3 ºC and 1-3 ºC, respectively, were observed, indicating POE and PEG were partial miscible.

*Table 5.1 Particle sizes of POE/PEG(MW 4,600) blend microspheres.*

| Polymer ratio (POE:PEG) | Average size (μm) | |
|---|---|---|
| | Volume mean diameter | Standard deviation |
| 100:0 | 88.56 | 15.00 |
| 95:5 | 92.16 | 15.36 |
| 90:10 | 82.96 | 15.95 |
| 80:20 | 74.64 | 11.51 |
| 70:30 | 68.00 | 14.41 |

*Table 5.2 Particle sizes of POE/PEO(MW 100,000) blend microspheres.*

| Polymer ratio (POE:PEO) | Average size (μm) | |
|---|---|---|
| | Volume mean diameter | Standard deviation |
| 100:0 | 88.56 | 15.00 |
| 95:5 | 85.05 | 17.96 |
| 90:10 | 85.02 | 18.06 |
| 80:20 | 84.38 | 18.89 |

*Table 5.3 Particle sizes of POE/PEO(MW 200,000) blend microspheres*

| Polymer ratio (POE:PEO) | Average size (μm) | |
|---|---|---|
| | Volume mean diameter | Standard deviation |
| 100:0 | 88.56 | 15.00 |
| 95:5 | 83.47 | 19.36 |
| 90:10 | 77.12 | 15.44 |
| 80:20 | 74.35 | 17.05 |

*Table 5.4a Inherent viscosity of POE:PEG(Mn 4,600)/CH2Cl2 solution.*

| Polymer blend (POE/PEG) ratio | Inherent viscosity (dL/g) |
|---|---|
| 95:5 | 2.81 |
| 90:10 | 2.90 |
| 80:20 | 2.69 |
| 70:30 | 2.54 |
| PEG 4,600 | 1.61 |

*Table 5.4b Inherent viscosity of POE:PEO(Mn100,000)/CH2Cl2 solution.*

| Polymer blend (POE/PEG) ratio | Inherent viscosity (dL/g) |
|---|---|
| 95:5 | 3.03 |
| 90:10 | 3.49 |
| 80:20 | 3.77 |
| PEO 100,000 | 7.76 |

*Table 5.4c Inherent viscosity of POE:PEO(Mn200,000)/CH2Cl2 solution.*

| Polymer blend (POE/PEO) ratio | Inherent viscosity (dL/g) |
|---|---|
| 95:5 | 3.05 |
| 90:10 | 3.59 |
| 80:20 | 4.74 |
| PEO 200,000 | 8.47 |

*Table 5.5a The effect of POE and PEG contents on polymer thermo-properties of the polymer blend.*

| Polymer weight ratio (POE:PEG) | *Tg* of POE, °C | *Tm* of PEG, °C |
|---|---|---|
| 100:0 | 102.90 | - |
| 0:100 | - | 60.10 |
| 95:5 | 104.75 | - |
| 90:10 | 104.10 | 56.98 |
| 80:20 | 104.84 | 59.00 |
| 70:30 | 104.89 | 59.00 |

*Table 5.5b The effect of POE and PEO(MW 100,000) contents on polymer thermo-properties of the polymer blend.*

| Polymer weight ratio (POE:PEO) | *Tg* of POE, ℃ | *Tm* of PEO, ℃ |
|---|---|---|
| 100:0 | 102.90 | - |
| 0:100 | - | 65.32 |
| 95:5 | 101.54 | 61.76 |
| 90:10 | 99.26 | 61.05 |
| 80:20 | 102.83 | 62.61 |

*Table 5.5c The effect of POE and PEO (MW 200,000) contents on polymer thermo-properties of the polymer blend.*

| Polymer weight ratio (POE:PEO) | *Tg* of POE, °C | *Tm* of PEO, °C |
|---|---|---|
| 100:0 | 102.90 | - |
| 0:100 | - | 65.50 |
| 95:5 | 102.45 | 62.24 |
| 90:10 | 99.00 | 61.60 |
| 80:20 | 103.20 | 61.87 |

### 5.1.3 PEG Contents in the Microspheres

The fabrication process involved a large volume of water and lasted about 4 hours. PEG is water-soluble. How much PEG was left in the microspheres? The $H^1$-NMR spectra of POE, PEG and POE/PEG blend microspheres are shown in Figures 5.1, 5.2 and 5.3, from which the PEG contents were calculated and listed in Tables 5.6a-5.6c. Clearly, a substantial fraction of PEG was lost into the aqueous phase during the microspheres fabrication process. PEO content of POE/PEO (MW 100,000 and 200,000) microspheres was much higher than that of POE/PEG (MW 4,600) blend microspheres. This is because the water-solubility of PEG with high molecular weights is lower, leading to more PEG incorporated in the microspheres. Increasing initial PEG loading yielded microspheres with higher PEG contents. PEG content would have a significant effect on BSA release. As reported, the presence of PEG can prevent the formation of BSA aggregates. On the other hand, during the course of *in vitro* release, the dissolution of PEG would leave pores and channels for the ease of BSA diffusion.

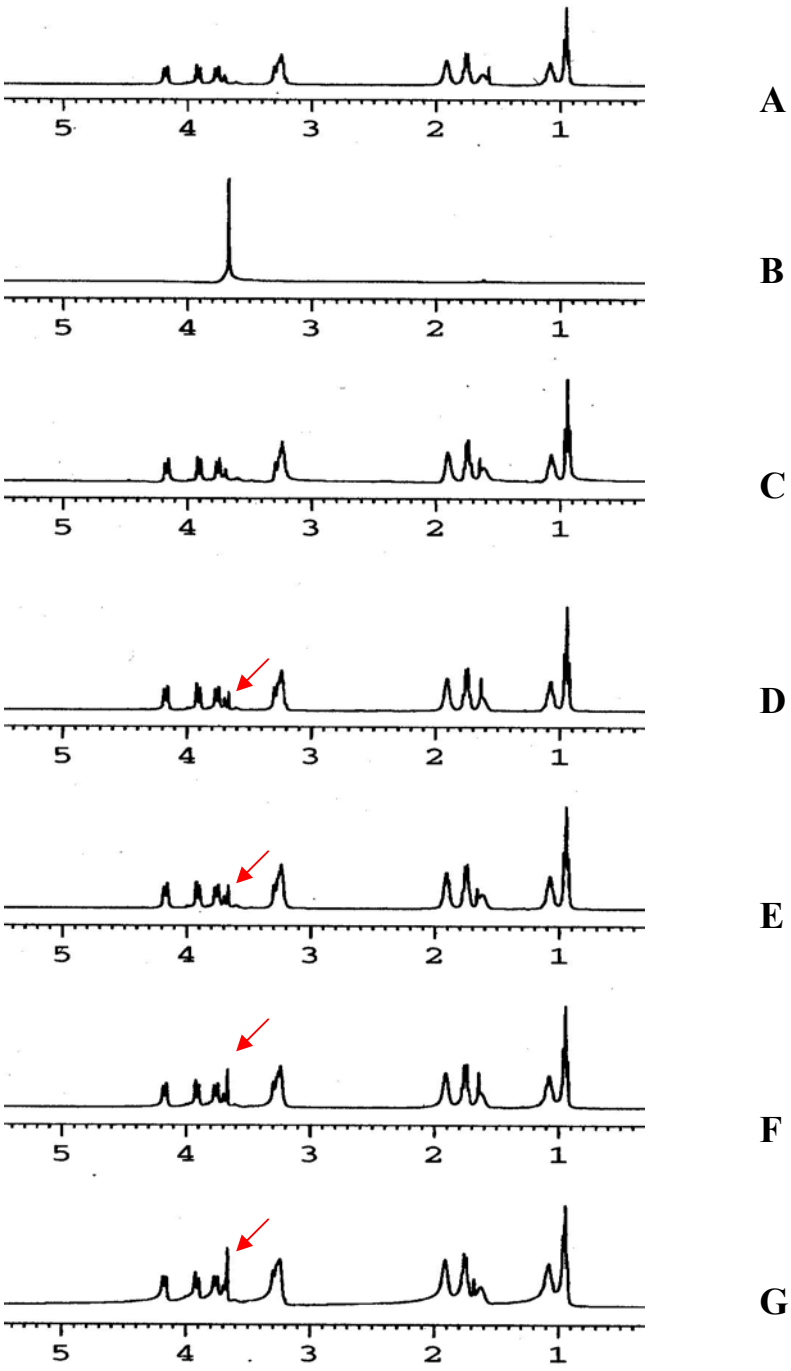



*Figure 5.1 $H^1$-NMR of: A, POE polymer; B, PEG (MW 4,600) polymer; C, POE microspheres; D, E, F and G, POE/PEG (MW 4,600) blend microspheres with PEG weight percentage 5%, 10%, 20% and 30% respectively.*

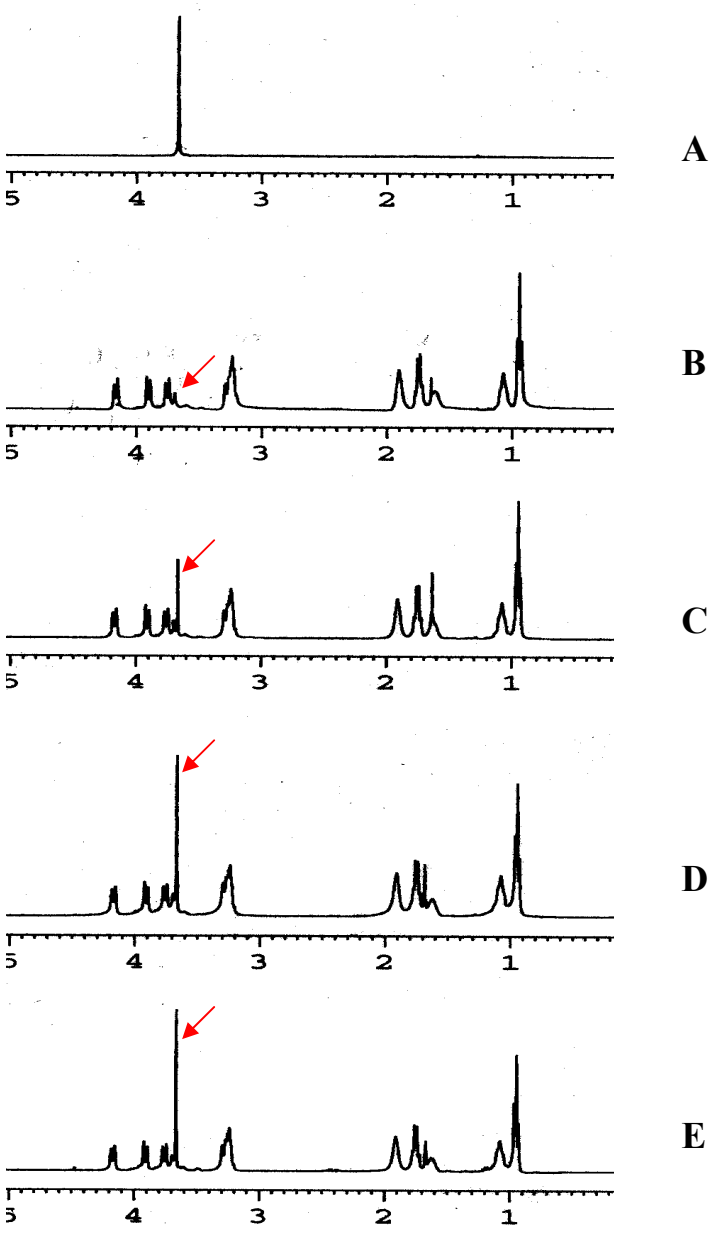



*Figure 5.2 NMR of: A, POE microspheres; B: PEO (MW 100,000) polymer; C, D, E: POE/PEO (MW 100,000) blend microspheres with PEO weight percentage 5%, 10% and 20% respectively.*

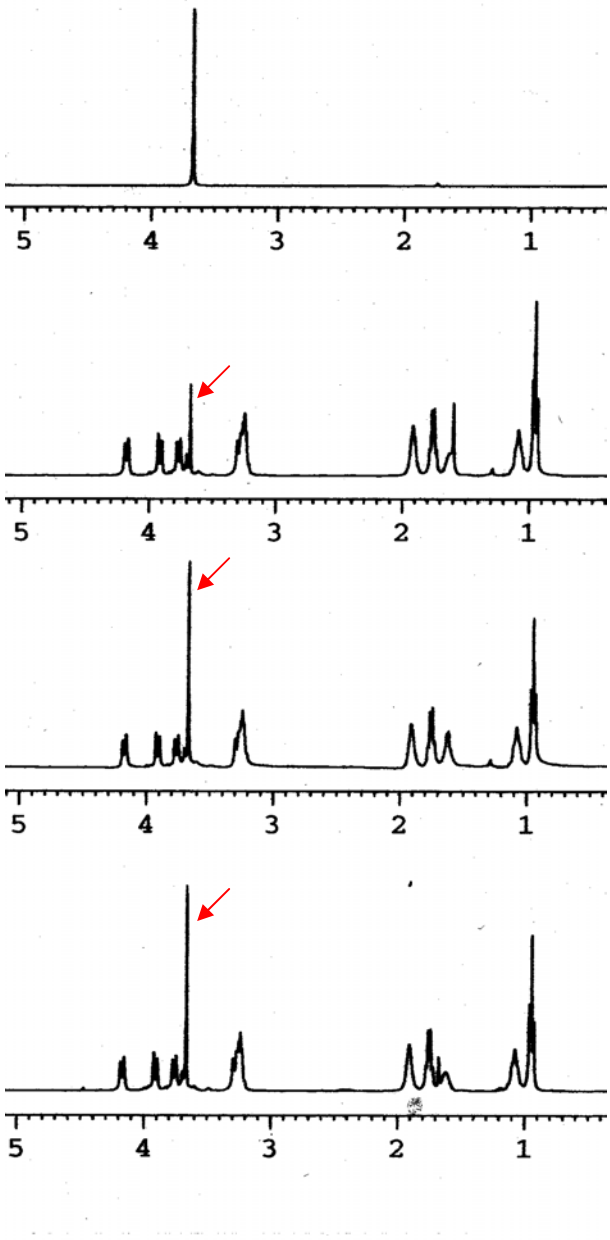



*Figure 5.3 NMR of: A, POE microspheres; B, PEO (MW 200,000) polymer; C, D, E, POE/PEO (MW 200,000) blend microspheres with PEO weight percentage 5%, 10% and 20% respectively.*

*Table 5.6a Percentage of PEG(MW 4,600) incorporated in microspheres*

| Initial PEG weight percentage, % | PEG weight percentage after fabrication, % |
|---|---|
| 5 | 0.8 |
| 10 | 1.2 |
| 20 | 1.7 |
| 30 | 3.5 |

*Table 5.6b Percentage of PEO(MW 100,000) incorporated in microspheres.*

| Initial PEG weight percentage, wt% | PEG weight percentage after fabrication |
|---|---|
| 5 | 2.2 |
| 10 | 2.4 |
| 20 | 6.8 |

*Table 5.6c Percentage of PEG(MW 200,000) incorporated in microspheres.*

| Initial PEG weight percentage, wt% | PEG weight percentage after fabrication, wt% |
|---|---|
| 5 | 1.1 |
| 10 | 5.8 |
| 20 | 5.8 |

## 5.2 Microspheres Morphology

### 5.2.1 Surface Morphology and Internal Structure of POE/PEG (MW 4,600) Blend Microspheres

POE/PEG(MW 4,600) blends yielded spherical particles with smooth surface when PEG weight percentage ranged from 5 to 20% (Figure 5.4). However, the surface of microspheres was rougher when 30% PEG was used. It was due to the loss of PEG into the external aqueous phase during the fabrication process. The internal structure of POE/PEG (MW 4,600) blend microspheres was shown in Figure 5.5. As reported in *Chapter 4*, the neat POE microspheres had a multi-vascular internal structure. However, POE/PEG blend microspheres with 5% PEG (MW 4,600) had more pores. When PEG content increased to 10%, the pores became smaller and more uniformly distributed. In addition, the inter-connection of pores was enhanced. In sharp contrast, macro-voids were observed within the micropsheres when PEG content further increased to 20% and 30%.

The internal structure of microspheres was basically attributed to the stability of the first emulsion. Figure 5.6 shows the demixing time of the first emulsion with various contents of PEG. With the introduction of PEG, the first emulsion became more stable, yielding smaller and uniformly-distributed pores within the microspheres. When PEG content was 20% or 30%, the demixing time was even longer. The first emulsion should be more stable. However, when the first emulsion was injected into the external aqueous phase, due to the hydrophilic nature of PEG, more water could be introduced into the system with a higher PEG content, leading to phase separation between polymer/solvent/non-

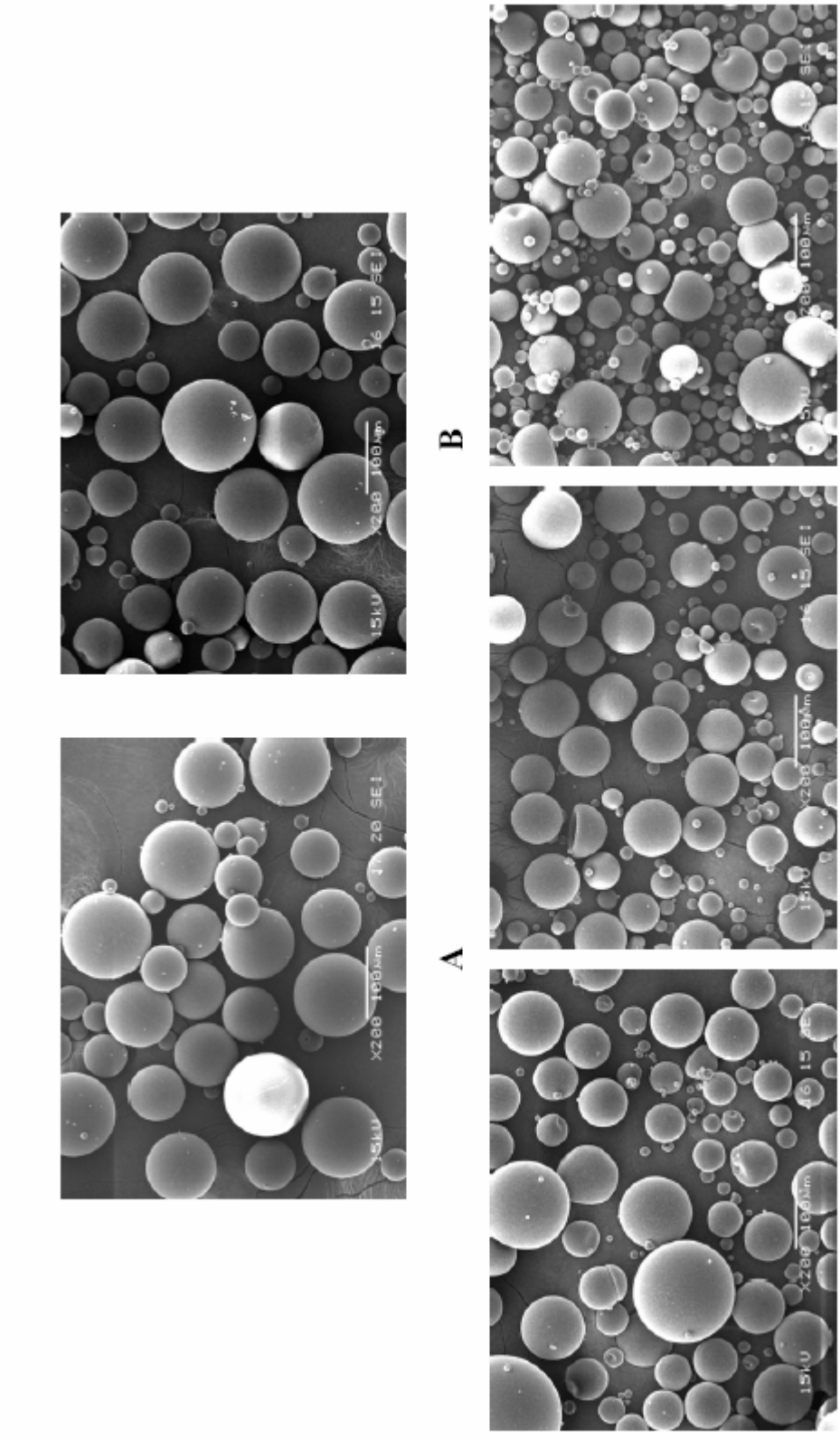



*Figure 5.4 Surface morphology of POE/PEG(MW 4,600) blend microspheres. PEG percentages of A, B, C, D and E are 0, 5%, 10%, 20% and 30% respectively. Size of bar is 100 μm.*

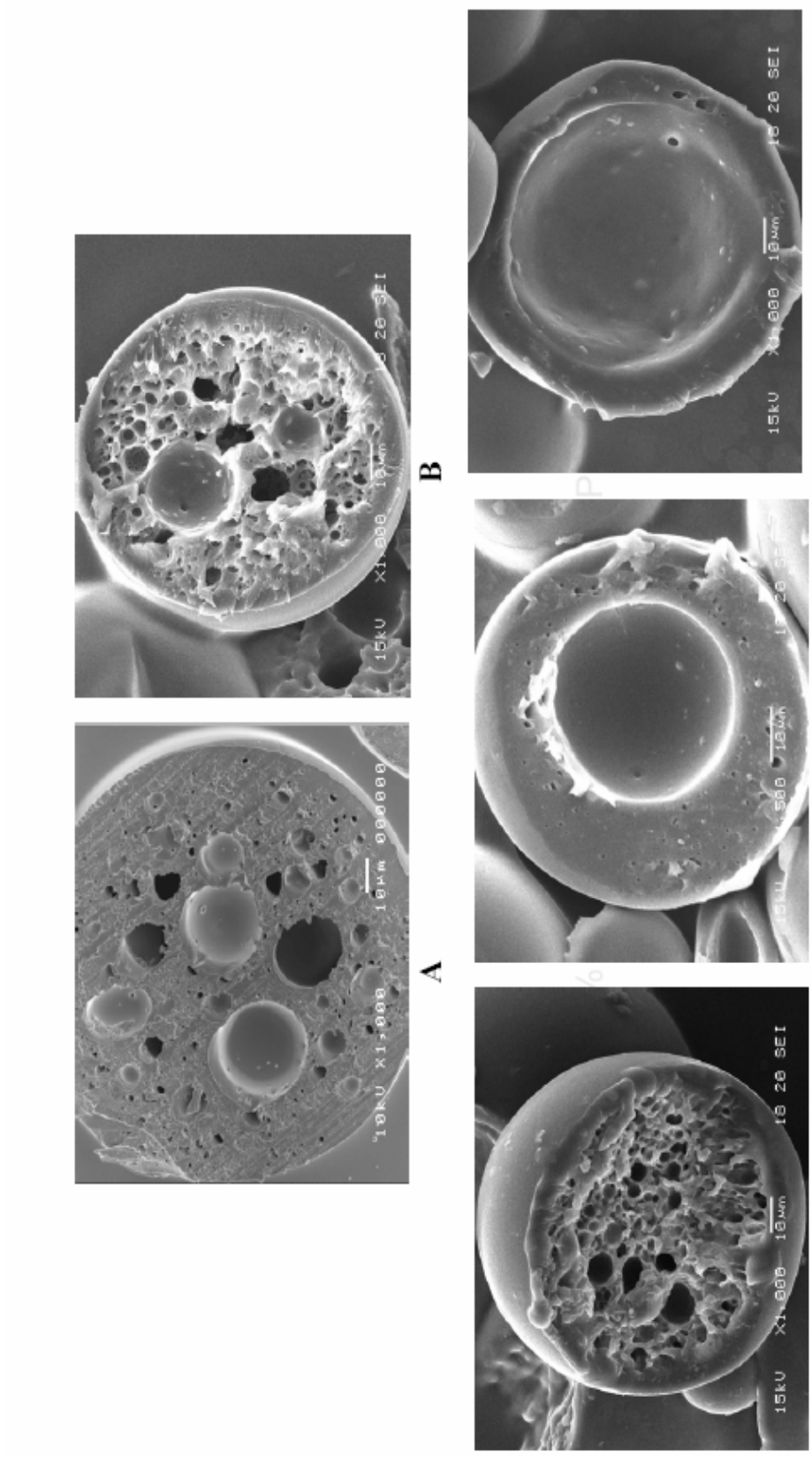



*Figure 5.5 Internal structure of POE/PEG(MW 4,600) blend microspheres. PEG percentages of A, B, C, D and E are 0, 5%, 10%, 20% and 30% respectively. Size of bar is 10 μm.*

solvent. Although a larger PEG content provided a more stable primary emulsion, greater water in-flux might result in the coalescence of internal water droplets during the fabrication process. Thus, macro-voids were observed within the microspheres made from 20% or 30% PEG.

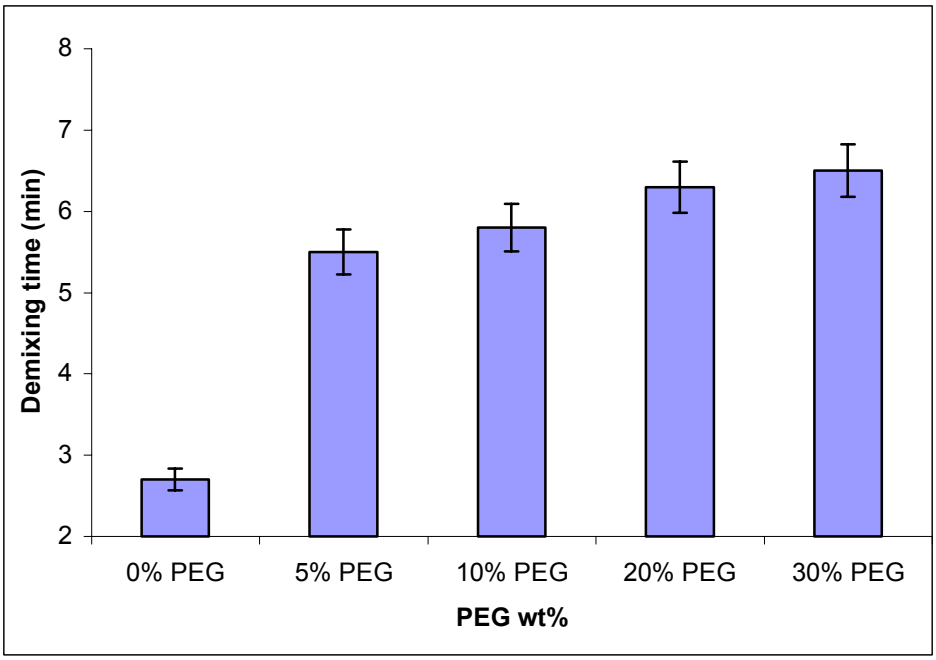



*Figure 5.6 Demixing time for first emulsion of POE/PEG(Mn 4,600) blend with various PEG content.*

Moreover, it was observed that the microspheres made from POE/PEG (MW 4,600) had a dense wall. It is probably because the introduction of PEG led to rapid solvent removal during the fabrication process. Theoretically, there are many parameters which can affect the solvent removal: polymer molecular weight and concentration, polymer crystallization, drug nature and method of incorporation (solid, liquid or suspension), organic solvent used, surfactant, fabrication temperature and stirring speed. In the present study, polymer molecular weight as well as blend ratio might be the key factor. It can be inferred that the formation of dense walls might affect BSA release.

### 5.2.2 Water Uptake

Water uptake is also an important parameter affecting polymer erosion and protein release. Figure 5.7 shows water absorption of the POE/PEG (MW 4,600) blend microspheres *versus* PEG content in PBS buffer (pH 7.4) at 37 ºC. The microspheres made with higher PEG contents had greater water uptake because of more porous structures created and the hydrophilic nature of PEG. It was also observed that it took about 7 days to reach the equilibrium of water absorption, which may be due to the hydrophobic nature of POE and the presence of wall.

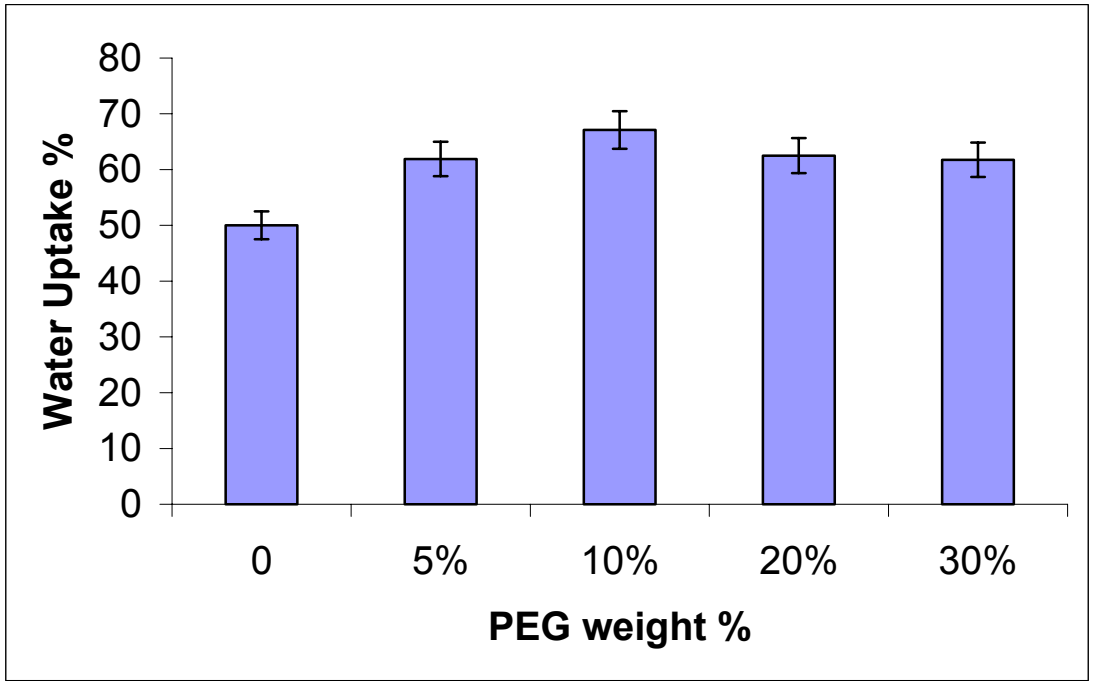



*Figure 5.7 Water uptake of POE/PEG(MW 4,600) blend microspheres after 7 days in vitro.*

### 5.2.3 Surface Morphology And Internal Structure of POE/PEO (MW 100,000 and 200,000) Blend Microspheres

Figures 5.8 and 5.9 show the surface morphologies of POE/PEO (MW 100,000 and 200,000) blend microspheres. With the increasing of PEO content, their surfaces appeared to be rougher and more porous. Possibly, it resulted from the dissolution of hydrophilic PEO. As we expected, during the fabrication process, PEO tended to dissolve in the external aqueous phase and leave pores. An increased PEG content produced rougher and more porous microspheres.

Figure 5.10 shows the internal structures of the microspheres. Interestingly, very porous internal structures were formed for the POE/PEG (MW 100,000) blend microspheres with various PEG contents. Except for POE/PEO(MW 100,000) microspheres with 5% PEG, where the pores size varied and the pores were separated, the microspheres with 10% or 20% PEG had a well inter-connected structure. Macro-voids were not observed even at high PEG contents. The phase separation did not occurred because the PEGs with higher molecular weights had lower water-solubility. The water content within the first emulsion droplets was not high enough to induce phase separation. The water uptake for POE/PEO (MW 100,000 and 200,000) blend microspheres is shown in Figure 5.11. Lower water uptake of the microspheres with PEG (MW 200,000) was due to their less porous structure.

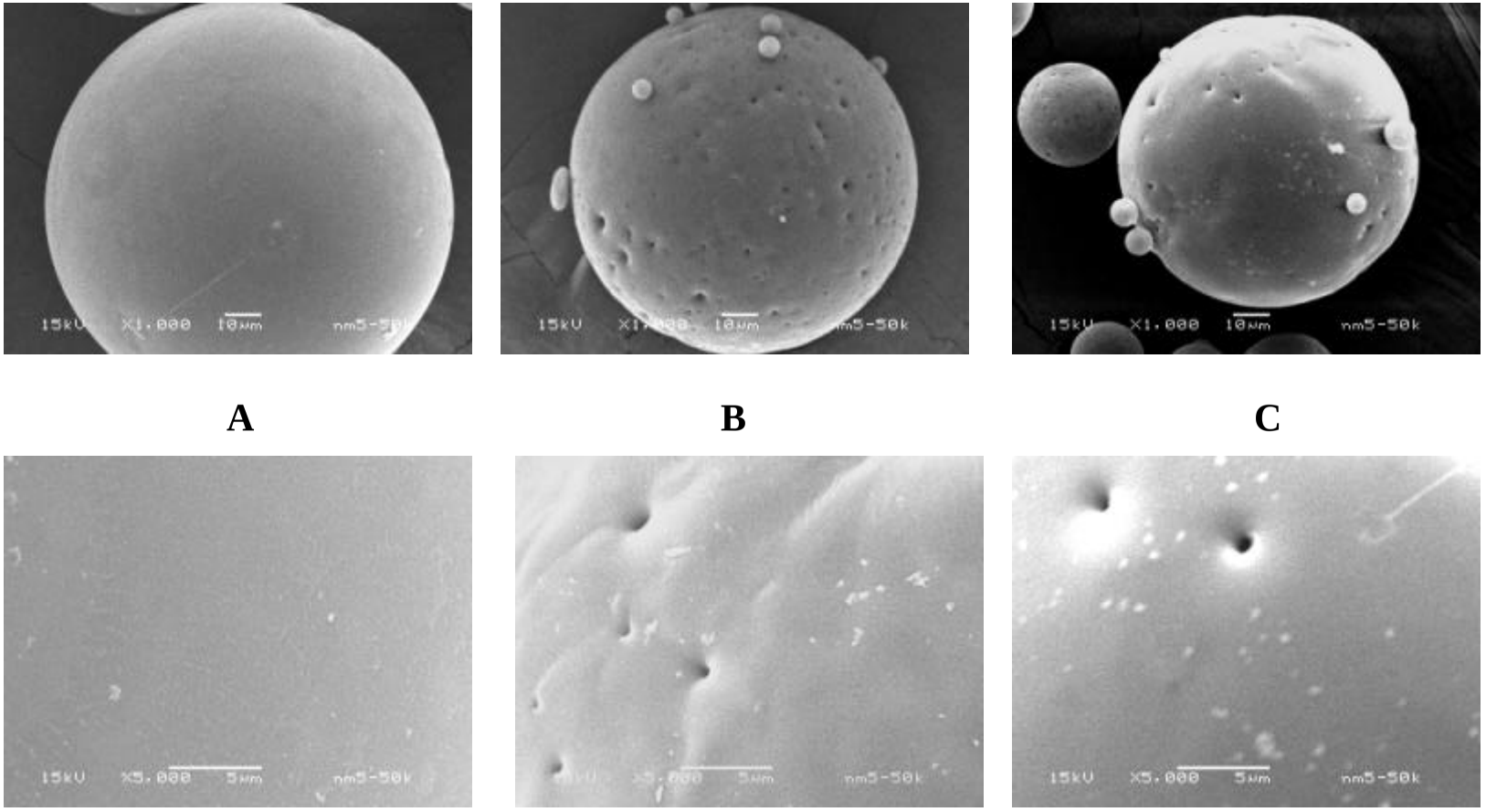



*Figure 5.8 Surface morphology of POE/PEO(MW 100,000) blend microspheres. A, B, C represent POE/PEO (MW 100,000) with 5%, 10% and 20% PEO. D, E and F represent surface morphology of POE/PEO(MW 100,000) microspheres with higher magnification.*

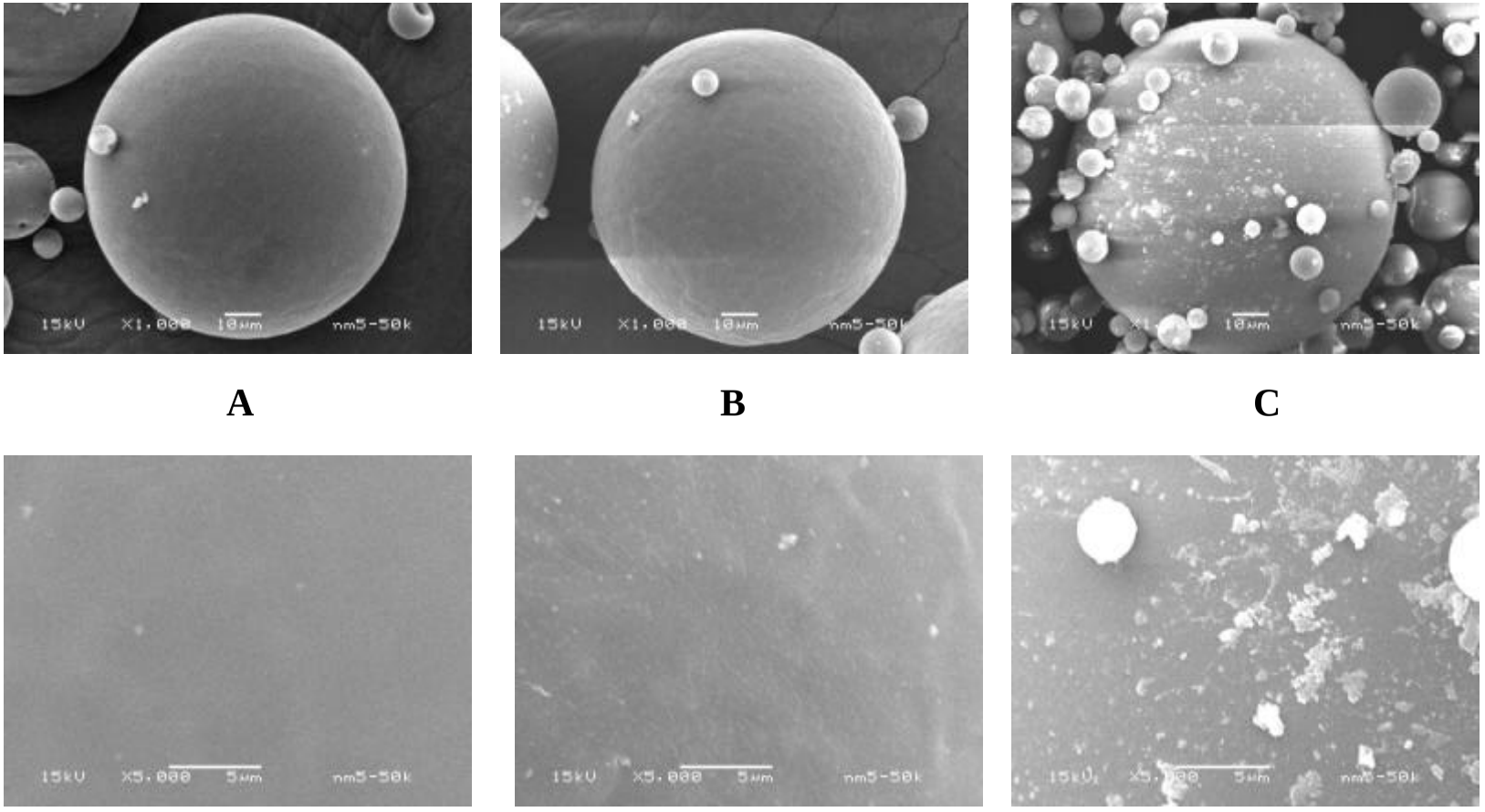



*Figure 5.9 Surface morphology of POE/PEO(MW 200,000) blend microspheres. A, B, C represent POE/PEO (MW 200,000) with 5%, 10% and 20% PEO. D, E and F represent surface morphology of POE/PEO(MW 200,000) microspheres with higher magnification.*

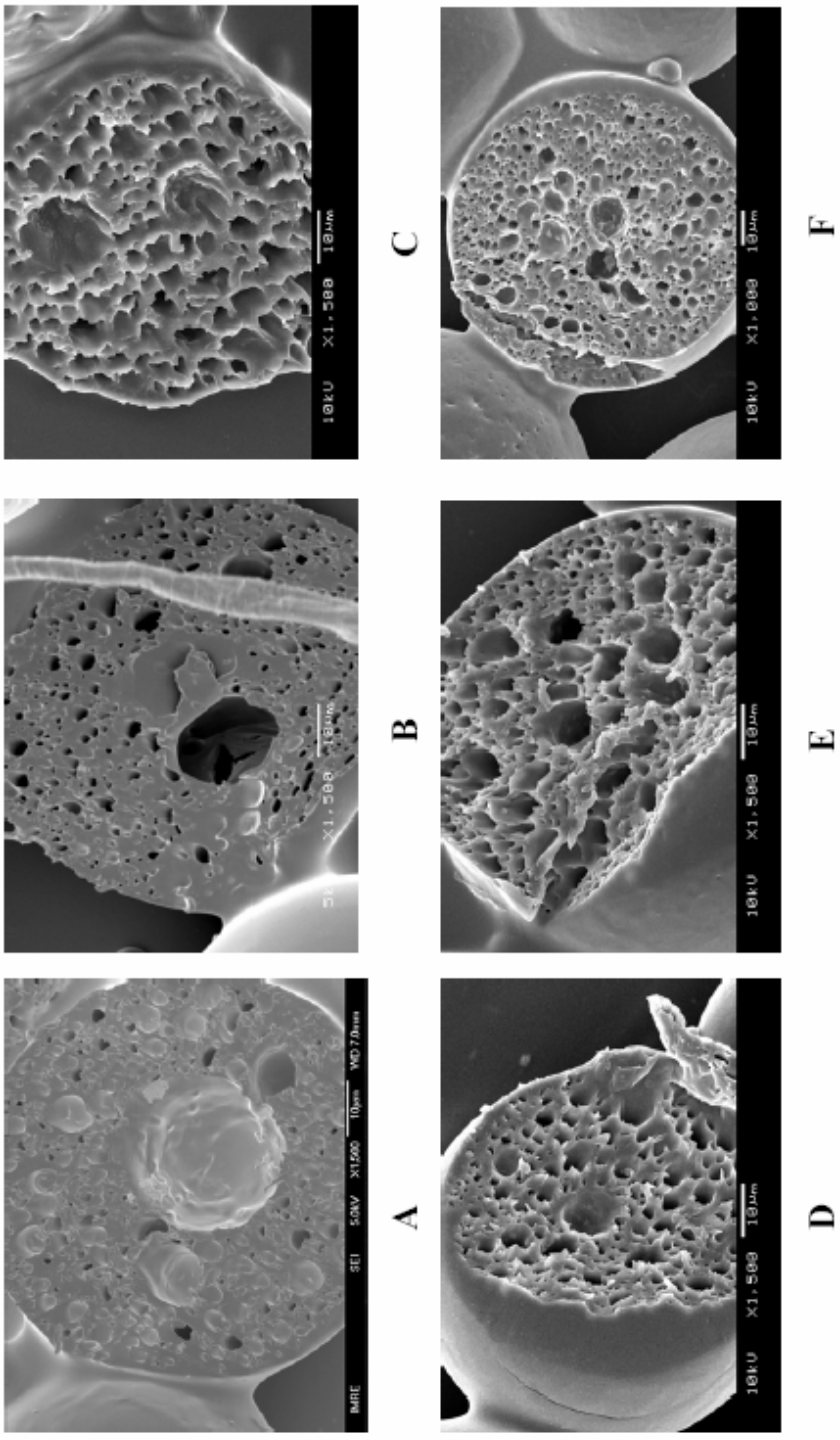



*Figure 5.10 Internal structure of POE/PEO(MW 100,000 and 200,000) blend microspheres. A, B, C represent POE/PEO (MW 100,000) with 5%, 10% and 20% PEO. D, E and F represent POE/PEO(MW 200,000) with 5%, 10% and 20% PEO. Size of bar is 10 μm.*

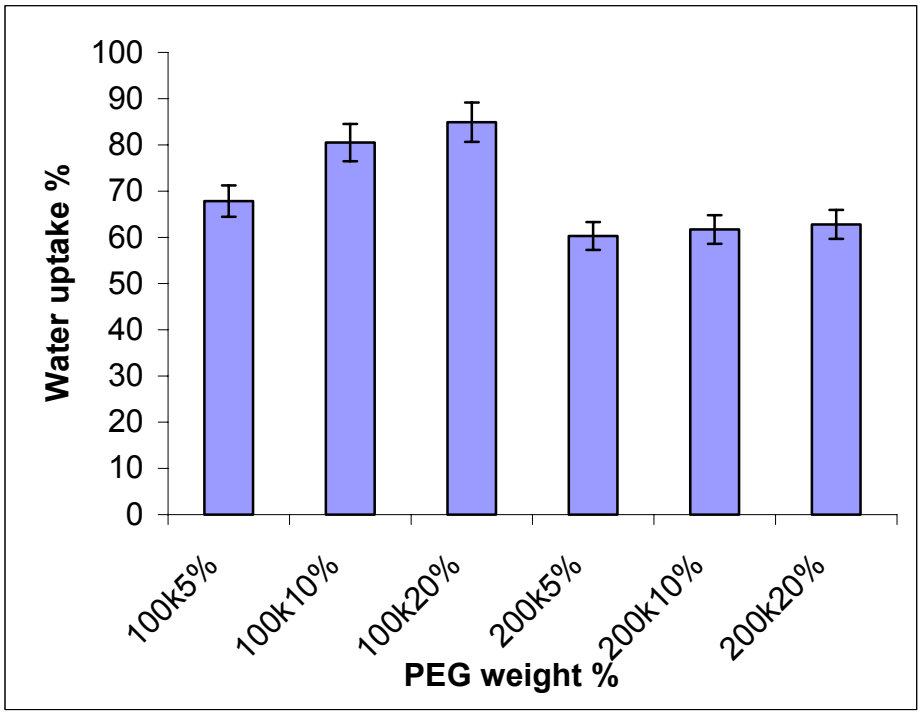



*Figure 5.11 Water uptake of POE/PEO(MW 100,000 and 200,000) blend microspheres after 7 days in vitro.*

## 5.3 BSA Encapsulation Efficiency

### 5.3.1 POE/PEG (MW 4,600) Blend Microspheres

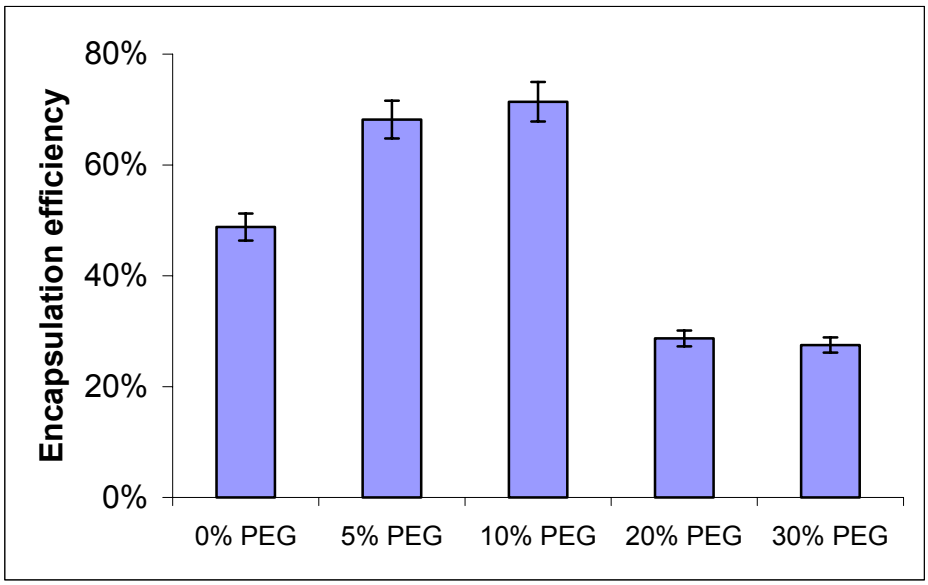



*Figure 5.12 Encapsulation efficiency of POE/PEG(MW 4,600) blend microspheres.*

Figure 5.12 illustrates BSA encapsulation efficiency of the POE/PEG (MW 4,600) blend microspheres with various PEG contents. From the figure, it can be observed that in the range from 0% to 10%, an increased PEG content led to increased BSA encapsulation efficiency. However, further increasing PEG content to 20%, the encapsulation efficiency dropped tremendously. For instance, 10% PEG yielded the highest encapsulation efficiency (70%), whereas it decreased to 30% at a PEG content of 20%.

M. K. Yeh et al. reported the protein loading efficiency was improved with the blending of PLGA with PEG compared to the microspheres made from PLGA alone [94]. However there were also results that described negative or insignificant effect of PEG blending on protein encapsulation. In the present study, more stable first

emulsion provided by the addition of PEG might account for the increased encapsulation efficiency of BSA. However, at high PEG contents (20 and 30%), greater water flux allowed more BSA to migrate towards the external aqueous phase, leading to lower encapsulation efficiency. On the other hand, the dissolution of more PEG could promote the loss of proteins. Furthermore, since a substantial amount of PEG was lost during the fabrication process, the overall polymer concentration was lower when higher contents of PEG were employed. Lower polymer concentration generally resulted in reduced encapsulation efficiency.

### 5.3.2 POE/PEO (MW 100,000 and 200,000) Blend Microspheres

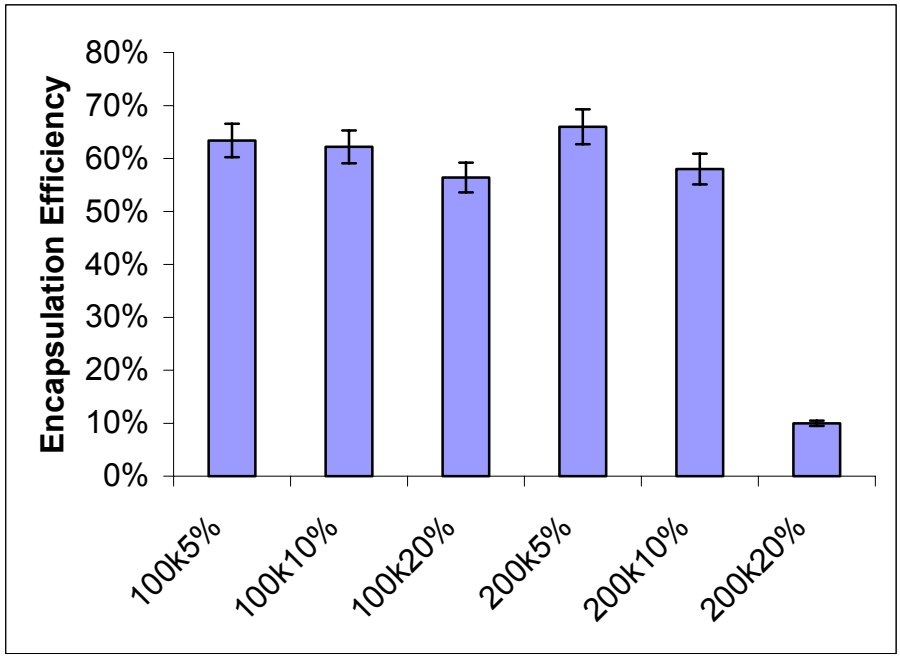



*Figure 5.13 Encapsulation efficiency of POE/PEO(MW 100,000 and 200,000) blend microspheres.*

Figure 5.13 shows BSA encapsulation efficiency of the microspheres made from POE/PEG (MW 100,000 and 200,000) blends. Similarly, an increased PEG content led to decreased encapsulation efficiency. Compared to POE/PEG (MW 100,000)

blend microspheres, BSA encapsulation efficiency of POE/PEO (200,000) blend microspheres was more dependent on PEG content.

## 5.4 BSA Release Profiles

### 5.4.1 POE/PEG (MW 4,600) Blend Microspheres

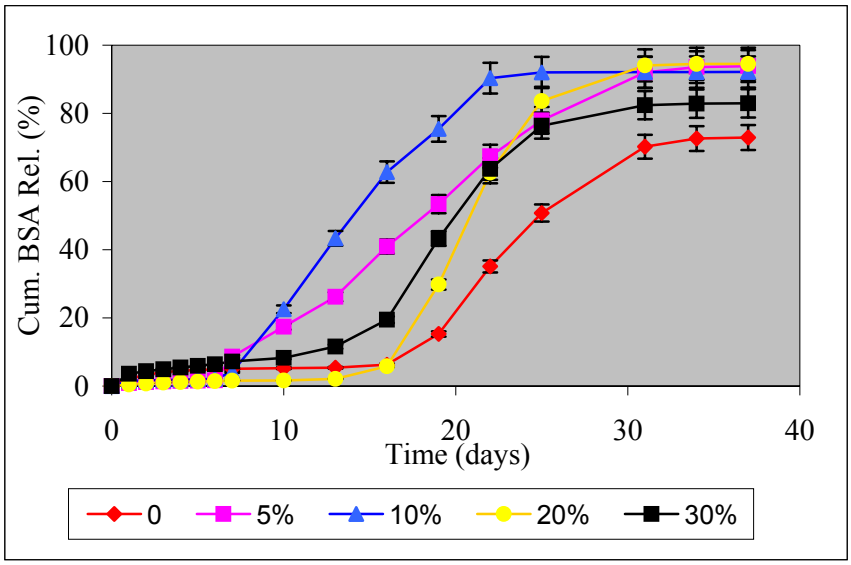



*Figure 5.14 BSA release profiles of POE/PEG(MW 4,600) blend microspheres. BSA loading is 10%.*

Figure 5.14 shows BSA release profiles from the POE/PEG blend microspheres. The release profiles were characterized by a lag phase followed by a sustained release of BSA. The dense wall surrounding the microspheres and hydrophobic nature of POE accounted for the existence of lag phase. The second phase (the sustained release phase) was due to BSA diffusion through the pores and polymer erosion. The introduction of PEG shortened the lag phase of BSA release, and achieved faster BSA release as well as more BSA released during the course of *in vitro* tests. The inter-connected porous structures enabled BSA to diffuse more easily. In addition, PEG remained in the microspheres promoted water penetration during the *in vitro* process.

Smaller particle size could be another reason. These results were consistent with what reported by M. K. Yeh et al. [94] and M. Wei et al. [127]. Interestingly, 5 and 10% PEG yielded a more sustained BSA release probably due to the more regularly distributed internal porous structure.

### 5.4.2 POE/PEG (MW 100,000 and 200,000) Blend Microspheres

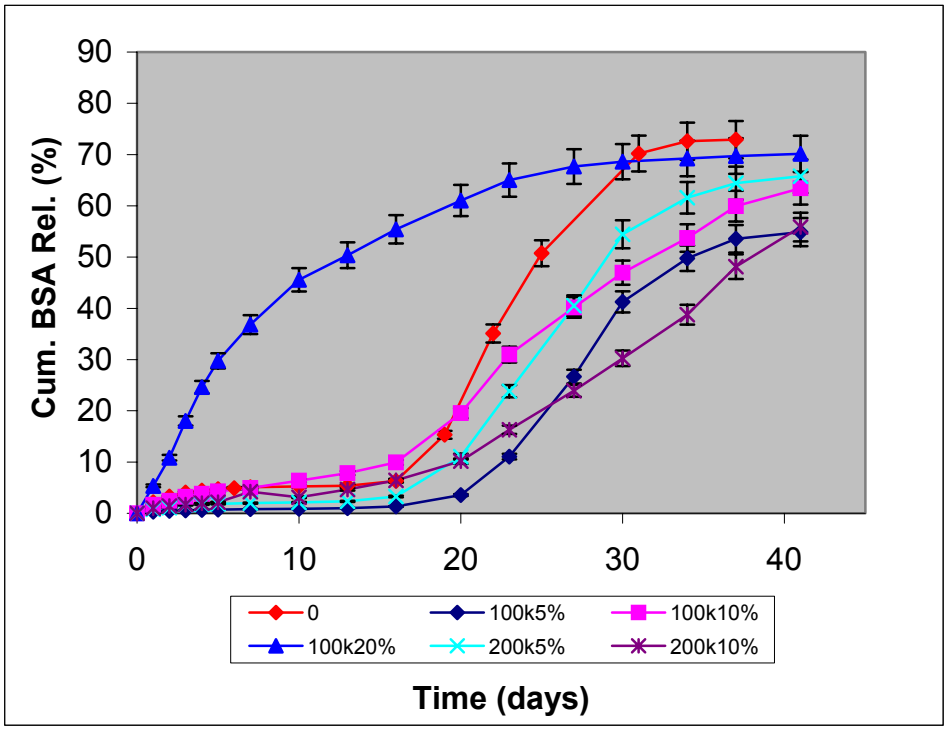



*Figure 5.15 BSA release profiles of POE/PEG (MW 100,000 and 200,000) blend microspheres. BSA loading is 10%.*

Release profiles of POE/PEG (MW 100,000 and 200,000) blend microspheres followed a very interesting pattern (Figure 5.15). It was largely determined by the particle size and internal structures.

A lag phase was still observed for the POE/PEO (MW 100,000 and 200,000) blend microspheres with 5% or 10% PEG. However, BSA release from the microspheres

made with 20% PEG (MW 200,000) was sustained over 3 weeks without lag phase. This is probably because the small particles (Table 5.2) provided greater surface area for BSA diffusion and dissolution.

# CHAPTER SIX

# CONCLUSIONS

## 6.1 POE-PEG-POE Microspheres

POE-PEG-POE with different molecular weights of PEG and various PEG contents have been employed to encapsulate protein using an improved W/O/W double emulsion evaporation/extraction method. This research work has demonstrated that spherical microparticles could be produced with POE-PEG-POE triblock copolymers having PEG contents of 5%, 10% and 20%. The protein distribution within the microspheres was uniform. Attaching a PEG block to POE polymer backbone yielded a more stable first emulsion (W/O) and thus a higher BSA encapsulation efficiency. However, PEG content and preparation conditions such as BSA loading, polymer concentration, PVA concentration in the external aqueous phase did not have significant influence on BSA encapsulation efficiency due to the stable first emulsion. Microsphere size could be tailored by the variation of polymer concentration. The absence of salt in the external aqueous phase yielded slightly higher encapsulation efficiency resulting from tighter and denser microsphere skins formed during the fabrication process.

Degradation of POE-PEG(5%)-POE and POE-PEG(10%)-POE triblock copolymers was characterised by an initial decrease in molecular weight and increase in polydispersity index during the first 2 weeks, which is followed by a constant value over 12 weeks *in vitro* while 90% mass was lost. However, POE-PEG(20%)-POE might possess a better compatibility between the POE and PEG blocks resulting in a relatively constant polydispersity index and molecular weight as well as a sustained weight loss. During the *in vitro* degradation, surface and internal morphologies of the microspheres did not change significantly. The microspheres eroded possibly from

their surfaces. The triblock copolymers with lower PEG contents did not show swelling but the POE-PEG(20%)-POE microspheres swells significantly after immersed in PBS buffer. Swelling of the microsphere matrices promoted release of the encapsulated proteins and leads to more complete proteins released. Protein release from the triblock copolymeric microspheres showed a biphasic pattern at low loading levels. The initial phase was dominated by diffusion and erosion for POE-PEG(5%)-POE and POE-PEG(10%)-POE. But for POE-PEG(20%)-POE, it depended on swelling, diffusion and erosion. The second phase might be controlled by properties of POE blocks and dense matrix structures. Intact BSA could remain within the matrices for 8 weeks in PBS buffer at pH 7.4, 37°C. A sustained protein release from POE-PEG(20%)-POE microspheres was achieved over two weeks at a high protein loading level.

PEG molecular weight did not have significant effect on release properties of BSA-loaded POE-PEG-POE microspheres. BSA release was much faster from POE-PEG-POE triblock copolymers with a POE composition of 1,2-PrD/1,2-PrD-diGL compared to the copolymers with a POE composition of CDM/TEG/TEG-diGL since 1,2-PrD is more flexible and hydrophilic. Therefore, the composition of POE should be well designed to yield a sustained and linear protein release.

## 6.2 POE/PEG Blend Microspheres

We fabricated microspheres from poly(ortho esters) (POE) and poly(ethylene glycol) (PEG) blends, BSA was entrapped using a double emulsion solvent extraction/evaporation technology to investigate the effect of blend ratio and PEG

molecular weight on microspheres property and release kinetics. NMR and DSC studies showed that a certain amount of PEG still remained in microspheres even after several hours' microspheres fabrication process. The remained PEG content in microspheres depended on the POE/PEG initial blend ratio as well as their molecular weights. The higher the PEG molecular weight, the more PEG remained in microspheres. DSC test revealed POE and PEG had partial miscibility. Swelling was observed for all the blend microspheres. Water uptake increased with the increasing of PEG content. All the blend microspheres had porous internal structure. It has also been observed that when the PEG(MW 4,600) content was as high as 20% and 30%, microcapsules could be formed. Definitely, the microcapsule internal structure would influence the protein release. These results demonstrate the feasibility of modulating the release profile of entrapped protein in biodegradable microspheres by adjusting the POE/PEG blend ratio and PEG molecular weight.

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# APENDICES

## LIST OF PAPERS FINISHED DURING PHD STUDY

1. Yi -Yan Yang, **Jin-Ping Wan**, Tai-Shung Chung, S. Ng, J. Heller. POE-PEG-POE triblock copolymeric microspheres containing protein. I. Preparation and Characterization, 75, 115-128, 2001, *Journal of Controlled Release*

2. **Jin-Ping Wan**, YY Yang, Tai-Shung Chung, S. Ng, J. Heller. POE-PEG-POE triblock copolymeric microspheres containing protein. II. Hydrolysis and Erosion Studies, 75, 129-141, 2001, *Journal of Controlled Release*

3. **Jin-Ping Wan**, Yi-Yan Yang, S. Ng & J. Heller, The degradation, swelling and erosion properties of new biodegradable POE-PEG-POE microspheres prepared by w/o/w technique (In progress, preparing to send to *Biomaterials*)

4. **Jin-Ping Wan,** Yi-Yan Yang, Tai-Shung Chung, Controlled protein delivery from POE/PEG blend microspheres (In progressing, preparing to send to Journal of Controlled Release)

5. **Jin-Ping Wan**, Yi-Yan Yang, Tai-Shung Chung, Steve Ng & Jorge Heller, Protein release from POE-PEG-POE tri-block copolymeric microspheres, Proceedings of the International Workshop on Advances in Materials Science and Technology, Singapore, 3-6 April 2000.

6. **Jin-Ping Wan**, Yi-Yan Yang, Tai-Shung Chung, Steve Ng & Jorge Heller**,** Properties and release profiles of POE-PEG-POE tri-block copolymeric microspheres, the 27$^{th}$ international symposium on controlled release of bio-active materials, Paris, France, 2000

7. **Jin-Ping Wan**, Yi-Yan Yang, Tai-Shung Chung, Steve Ng and Jorge Heller, Polymer erosion and protein release design of POE-PEG-POE triblock copolymeric microspheres, the 28th international symposium on controlled release of bio-active materials, San Diego, USA, June 2001

8. Yi-Yan Yang, **Jin-Ping Wan**, Tai-Shung Chung, Steve Ng and Jorge Heller, Design on protein release profile of biodegradable POE-PEG-POE microspheres. International conference on materials for advanced technologies, 1-6 July 2001, Singapore

9. **Jin-Ping Wan**, Yi-Yan Yang, S. Ng, T. S. Chung and J. Heller, Characterization and protein release study of poly(ortho ester) and its block copolymeric microspheres, Invited talk in 25th Australasian Polymer Symposium, 10-13 Feb, 2002, University of New England, Armidale, Australia

10. Yi-Yan Yang, **Jin-Ping Wan**, Cherng-Wen Tan, Poly (ortho ester) and poly(ortho ester)-poly(ethylene glycol)-poly(ortho ester) microspheres for protein delivery, Invited talk in *Particles*, 2002